

APPLICATION  
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TITLE: NON-NUCLEAR EFFECTS OF THYROID HORMONE

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## NON-NUCLEAR EFFECTS OF THYROID HORMONE

### CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority from U.S. provisional patent application serial no. 60/214,572, filed on June 28, 2000, which is incorporated herein by reference in its entirety.

### TECHNICAL FIELD

This invention relates to thyroid hormone receptors, and more particularly to non-nuclear effects of thyroid hormone.

### BACKGROUND

Thyroid hormone (TH) has diverse effects on mammals, including effects on the neonatal and adult brain. In the developing animal, thyroid hormone regulates various events such as neuronal processing, glial cell proliferation, myelination, and neurotransmitter enzyme synthesis. The metabolically active form of thyroid hormone, 3, 5, 3'-triiodothyronine (T3), acts by binding to two receptors; TR $\alpha$ 1 and TR $\beta$ 1. These two receptors are encoded by the c-erbA $\alpha$  locus. Two truncated receptor transcripts have been identified that are also transcribed from the c-erbA $\alpha$  locus;  $\Delta$ TR $\alpha$ 1 and  $\Delta$ TR $\alpha$ 2 (Chassande et al., 1997, Mol. Endocrinol. 11:1278-1290). Neither truncated receptor has a DNA binding region and  $\Delta$ TR $\alpha$ 1 has been shown to antagonize T3-induced transcriptional activation.

Type II iodothyronine 5'-deiodinase (D2) is the key enzyme in the pathway that mediates the conversion of intracellular thyroxine (T4) to 3, 5, 3'-triiodothyronine (T3). D2 concentration can be mediated by thyroid hormone concentration and is regulated by enzyme inactivation. The D2 activity appears to be more sensitive to T4 than T3. The degradation of the enzyme is energy-dependent and apparently requires the structural integrity of the actin cytoskeleton, i.e., is regulated at least in part by actin-based endocytosis. p29 is the substrate binding subunit of D2. T4 induces inactivation of D2 and initiates the binding of p29 to F-

5 actin. The bound p29 is transported to an endosomal pool followed by dissociation of the F-actin-p29 complex (Farwell et al., 1993, J. Biol. Chem. 268: 5055-5062).

### SUMMARY

The present invention relates to the effects of thyroid hormone that are mediated by non-nuclear mechanisms. In particular, the invention relates to methods of identifying  
10 compounds that interact with a  $\Delta\text{TR}\alpha 1$  polypeptide and/or  $\Delta\text{TR}\alpha 2$  polypeptide ( $\Delta\text{TR}\alpha 1$ ;  $\Delta\text{TR}\alpha 2$ ). The invention also includes transgenic animals with altered or missing  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$ . Such animals are useful for identifying new targets for drug discovery.

Although  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  do not bind to 3, 5, 3'-triiodothyronine (T3), they do bind with high affinity to 3, 3', 5'-triiodothyronine (reverse T3; rT3) and that binding can be  
15 displaced by thyroxine (T4) and rT3 (Example 2). The invention relates to the discovery that the association of myosin V with p29 vesicles is dependent on thyroid hormone, e.g., T4 and rT3, bound to a  $\Delta\text{TR}\alpha 2$ , and that stable complexes between p29, several synaptic vesicle proteins and myosin V can be isolated on actin fibers. Thus, T4, rT3, and certain analogs are useful for regulating actin-based endocytosis, especially movement of synaptic vesicles.

The invention features a method of assaying the functionality of a translation product of a mutant  $\Delta\text{TR}\alpha 2$  gene in a cell. The method includes binding a labeled ligand for a  
20  $\Delta\text{TR}\alpha 2$  polypeptide to the translation product in a cell and measuring the amount, location, or rate of transit of the ligand in the cell. An increase in the amount, location, or rate of transit of the ligand in the cell compared to that in a cell that does not comprise a mutant  $\Delta\text{TR}\alpha 2$  gene indicates an increase in functionality of the translation product. A decrease in  
25 the amount location, or rate of transit of the ligand in the cell compared to a cell that does not comprise a mutant  $\Delta\text{TR}\alpha 2$  gene indicates a decrease in the functionality of the translation product. The ligand can be, e.g., a flavone, an aurone, or a T4 analog.

The invention includes an inhibitor of  $\Delta\text{TR}\alpha 2$  expression or activity. The inhibitor  
30 can be, e.g., a flavone, an aurone, or a T4 analog.

The invention also features a method of identifying a candidate compound that modulates  $\Delta\text{TR}\alpha 2$  activity by obtaining a  $\Delta\text{TR}\alpha 2$  polypeptide, contacting the  $\Delta\text{TR}\alpha 2$  with a test compound, assaying for binding of the test compound to  $\Delta\text{TR}\alpha 2$ , such that binding

5 indicates that the test compound that binds to the  $\Delta\text{TR}\alpha 2$  polypeptide is a candidate compound that modulates  $\Delta\text{TR}\alpha 2$  activity. The test compound can be, e.g., a flavone, an aurone, or a T4 analog.

In another aspect, the invention provides a method of identifying a candidate compound that modulates  $\Delta\text{TR}\alpha 2$  activity. This method includes obtaining a  $\Delta\text{TR}\alpha 2$  polypeptide bound to a  $\Delta\text{TR}\alpha 2$  ligand, contacting the  $\Delta\text{TR}\alpha 2$  bound to the  $\Delta\text{TR}\alpha 2$  ligand with a test compound, and measuring the displacement of the  $\Delta\text{TR}\alpha 2$  ligand from the  $\Delta\text{TR}\alpha 2$  polypeptide, such that displacement indicates that the a test compound is a candidate compound that modulates  $\Delta\text{TR}\alpha 2$  activity. The test compound can be, e.g., a flavone, an aurone, or a T4 analog.

15 The invention also includes a method of identifying a candidate compound that modulates  $\Delta\text{TR}\alpha 2$  activity. This method includes the steps of obtaining a test sample containing a  $\Delta\text{TR}\alpha 2$ , incubating the test sample with a test compound, and assaying the test sample containing the test compound for an alteration in type II 5' deiodinase (D2) activity, such that a test compound that alters D2 activity when compared to a test sample that was not incubated with the test compound is a candidate compound. In this method, the test compound may decrease the amount of D2 activity. The test compound can be, e.g., a flavone, an aurone, or a T4 analog.

25 The invention also features a method of identifying a candidate compound that modulates  $\Delta\text{TR}\alpha 2$  activity which includes the steps of obtaining a test sample containing a  $\Delta\text{TR}\alpha 2$ , performing an actin binding assay with the test sample in the presence of a test compound, such that a test compound that alters the binding of p29 vesicles to F-actin when compared to a test sample that was not incubated with the test compound is a candidate compound. The test compound can be, e.g., a flavone, an aurone, or a T4 analog.

30 The invention includes a compound identified by the any of the methods described above. The invention also includes an inhibitor of  $\Delta\text{TR}\alpha 2$  expression or activity.

Other aspects of the invention are methods of treating a subject who has a neurologic disorder or a psychiatric disorder (e.g., a mood disorder or depression) by administering to the subject a therapeutically effective amount of a  $\Delta\text{TR}\alpha 2$  ligand.

5 The invention also features an isolated nucleic acid molecule that includes a  $\Delta$ TR $\alpha$ 2 targeting construct that contains a DNA sequence homologous to sequences encoding a mouse  $\Delta$ TR $\alpha$ 2, such that when the construct is introduced into a non-human animal (e.g., a mouse) cell or an ancestor of the animal cell at an embryonic stage, and the construct-derived sequences are incorporated into an endogenous TR $\alpha$  gene, the cell does not express  $\Delta$ TR $\alpha$ 2  
10 in significant amounts (e.g., not more than 75 %, 50%, 25%, 10%, or 5% of the level of expression in a cell or animal having a wild type gene). The invention includes a vector containing this nucleic acid. The construct can contain a nucleic acid sequence that is homologous to intron 7 of a mouse TR $\alpha$  gene or a nucleic acid sequence that is homologous to exon 10 of a mouse TR $\alpha$  DNA sequence. In some aspects of the invention, introduction of  
15 the construct into the cell disrupts the AP1, ctf, GR, SP1, or ets1 sequence of intron 7. The isolated nucleic acid molecule can also include a gene selection cassette.

The invention features a transgenic, non-human animal whose germ cells and somatic cells include a mutated TR $\alpha$  gene, the mutation being sufficient to inhibit binding of thyroxine (T4) to  $\Delta$ TR $\alpha$ 2 transcribed from the gene. The mutated gene is introduced into the  
20 non-human animal or an ancestor of the animal at an embryonic stage, such that the animal, if homozygous for the mutation, has impaired motor function. The non-human animal can be a mouse, a rat, a goat, a sheep, or a pig. The invention includes a cell derived from the transgenic animal. The cell can be an astrocyte or other neuronal cell type. In such transgenic animals, the TR $\alpha$  gene can be mutated in intron 7 or in exon 10.

25 Another aspect of the invention features a transgenic non-human animal whose somatic and germ cells include a disrupted TR $\alpha$  gene, the disruption being sufficient to inhibit the binding of T4 to a  $\Delta$ TR $\alpha$ 1 or  $\Delta$ TR $\alpha$ 2 translation product of the TR $\alpha$  gene and the disrupted gene was introduced into the animal or an ancestor of the animal at an embryonic stage. Such an animal, if homozygous for the disrupted gene, has impaired motor function.  
30 The animal can be a rodent (e.g., a mouse or a rat), a goat, a pig, or a sheep. The disruption in such an animal can include a mutation in intron 7 or exon 10 of the TR $\alpha$  gene. The disruption can include a deletion of all or a part of intron 7 of the TR $\alpha$  gene or a deletion of all or part of exon 10 of the TR $\alpha$  gene.

5 A "transgene" is any piece of DNA that is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from that cell or progeny of the organism. Such a transgene may include a gene that is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene or part of a gene that is homologous with an endogenous gene of the organism.

10 A cell that is "transgenic" is one which includes a DNA sequence inserted by artifice into a cell to become part of the genome of the organism which develops from that cell or progeny of the organism. As used herein, the transgenic organism can be an animal, generally a mammal (e.g., a rodent such as a mouse or rat), and the DNA (transgene) is inserted into the nuclear genome.

15 A "transgenic animal" is an animal which includes a transgene. In general, the transgene is inserted into an embryonal cell and becomes a part of the genome of the animal which develops from that cell, or an offspring of such an animal. The transgene may introduce a heterologous DNA sequence into the embryonal cell or introduce an alteration such as a deletion, insertion, or substitution of an endogenous DNA sequence (e.g., by homologous recombination). In the transgenic animals described herein, the transgene causes cells to express an altered form of  $\Delta TR\alpha 1$  or  $\Delta TR\alpha 2$ . Such animals include those produced using methods such as homologous recombination. In general, the animals produced by the transgenic technology of the invention are mammals although any animal that can be produced by such technology is encompassed by the invention. Mammals used for the invention include non-human primates, sheep, goats, horses, cattle, pigs, rabbits, and rodents such as guinea pigs, hamsters, rats, gerbils, and mice.

25 As used herein, a "homologously recombinant animal" is a non-human animal, e.g., a mammal, such as a mouse, in which an endogenous gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal, e.g., an embryonic cell of the animal, prior to development of the animal.

30 A " $\Delta TR\alpha 1$  ligand" or " $\Delta TR\alpha 2$  ligand" is a compound that binds to a  $\Delta TR\alpha 1$  or  $\Delta TR\alpha 2$ , respectively. In some embodiments such a ligand binds to a  $\Delta TR\alpha 1$  or  $\Delta TR\alpha 2$  with an affinity of greater than or equal to  $10^{-8}$  Mol/L.

5 A molecule that "specifically binds" is a molecule that binds to a particular entity, e.g., a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ , but which does not substantially recognize or bind to other molecules in a sample, e.g., a biological sample, which includes that particular entity, e.g., a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ .

10 The terms  $\Delta\text{TR}\alpha 1$  "candidate compound" or  $\Delta\text{TR}\alpha 2$  "candidate compound" refer to compounds that interact with or affect the activity of a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ . Such candidate compounds may be identified, e.g., by their ability to bind to one of the receptors, by their ability to displace a bound ligand from the receptor, by indirect assays such as ability to alter D2 activity when the candidate compound is incubated with the receptor in a D2-containing preparation, or by this ability to affect the association of myosin V with p29 vesicles.

15 Candidate compounds may also be ligands.

A "test compound" is a compound used in the methods of the invention that is tested for its qualifications as a candidate compound.

A "homologous sequence" is a sequence with identity to a reference sequence. Calculations of homology (i.e., sequence identity) between sequences are performed as follows.

20

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In an embodiment, the length of a reference sequence aligned for comparison purposes (e.g., when aligning a second sequence to a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  amino acid sequence) is at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the length of the reference sequence. The length of the reference sequence can also be 100%. The reference sequence can be a full-length amino acid sequence of  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  or a partial sequence, e.g., a domain, intron, or exon (such as intron 7 or exon 10 of a mouse  $\text{TR}\alpha$  sequence). In an embodiment, the length of a reference sequence aligned for comparison purposes (e.g., when aligning a second sequence to a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  nucleic acid sequence) is at least 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the length of the reference sequence. The length of the reference nucleic acid sequence can also be 100%. The reference sequence can be a full-

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5 length nucleic acid sequence encoding a  $\Delta TR\alpha 1$  or  $\Delta TR\alpha 2$  or a partial sequence, e.g., a  
sequence that codes for a domain, intron, or exon such as intron 7 or exon 10. For some  
purposes, e.g., homologous recombination, the nucleic acid sequence may be a genomic  
sequence (e.g., include intron sequences). The amino acid residues or nucleotides at  
corresponding amino acid positions or nucleotide positions are compared. When a position  
10 in the first sequence is occupied by the same amino acid residue or nucleotide as the  
corresponding position in the second sequence, then the molecules are identical at that  
position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or  
nucleic acid "homology"). The percent identity between the two sequences is a function of  
the number of identical positions shared by the sequences, taking into account the number of  
15 gaps, and the length of each gap, which need to be introduced for optimal alignment of the  
two sequences.

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The comparison of sequences and determination of percent identity between two  
sequences can be accomplished using a mathematical algorithm. In a preferred embodiment,  
the percent identity between two amino acid sequences is determined using the Needleman  
20 and Wunsch ((1970) J. Mol. Biol. 48:444-453 ) algorithm which has been incorporated into  
the GAP program in the GCG software package (available at <http://www.gcg.com>), using  
either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or  
4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent  
identity between two nucleotide sequences is determined using the GAP program in the GCG  
25 software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a  
gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. Another set of  
parameters (e.g., that can be used if the practitioner is uncertain about what parameters  
should be applied to determine if a molecule is within a sequence identity or homology  
limitation of the invention) are a Blossum 62 scoring matrix with a gap penalty of 12, a gap  
30 extend penalty of 4, and a frameshift gap penalty of 5.

The percent identity between two amino acid or nucleotide sequences can be  
determined using the algorithm of E. Meyers and W. Miller ((1989) CABIOS, 4:11-17)  
which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight  
residue table, a gap length penalty of 12 and a gap penalty of 4.



5 The nucleic acid and protein sequences described herein can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences (such as human  $\Delta\text{TR}\alpha 1$ ,  $\Delta\text{TR}\alpha 2$ , or myosin V amino acid or nucleic acid sequences). Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST  
10 nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to nucleic acid molecules useful in the invention (such as human  $\Delta\text{TR}\alpha 1$ ,  $\Delta\text{TR}\alpha 2$ , or myosin V). BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to protein molecules useful in the invention. To obtain gapped  
15 alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

20 The terms "sufficiently identical" or "substantially identical" are used herein to refer to a first amino acid or nucleotide sequence that contains a sufficient or minimum number of identical or equivalent (e.g., with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences have a common structural domain or common functional activity. For example, amino acid or nucleotide sequences that contain a common structural domain  
25 having at least about 60%, or 65% identity, likely 75% identity, more likely 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identity are defined herein as sufficiently or substantially identical.

30 A mutant  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  gene encodes a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  polypeptide that includes a change in comparison to the wild-type amino acid sequence. In general, these changes arise from genetic engineering (e.g., by transgenic methods). These changes also include naturally occurring mutations and alleles.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can  
35 be used in the practice or testing of the present invention, suitable methods and materials are

5 described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

10 Among the advantages of the present invention are new methods of identifying candidate compounds that affect the non-nuclear effects of thyroid hormone, e.g., those involving  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$ . Such compounds may be useful in treatments for disorders that involve such non-nuclear effects. The present invention also provides methods of treatment for disorders of the nervous system and psychiatric disorders such as depression, e.g., with compounds that bind to  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$ .

15 The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

### DESCRIPTION OF DRAWINGS

20 Figure 1 is a schematic representation of the general structure of flavones and examples of structures of candidate compounds.

Figure 2 is a schematic representation of the general structure of aurones.

Figure 3 is a schematic representation of the structure of iodothyronine.

Figure 4 is a schematic representation of the general structure of phloretin.

25 Figure 5 is a diagram of myosin V truncation mutants and indicates the percentage of p29 binding to myosin V competed out by each mutant peptide.

Figure 6 is a diagram of intron 7 of a  $\text{TR}\alpha$  gene.

Figure 7 is a diagram of a scheme for preparing the 5' and 3' arms of a  $\text{TR}\alpha$  gene for deletion of intron 7 promoters.

30 Figure 8 is a diagram of a scheme for replacing exon 10 of a  $\text{TR}\alpha$  gene with a neo<sup>r</sup> selection gene.

Figure 9 is a graph illustrating the antibody inhibition of TH-dependent p29 vesicle binding to actin fibers *in vitro*. Data are expressed as the percent maximal binding and are the means  $\pm$  SE of triplicate determinations.

5 Figure 10 is a graph illustrating the effects of acute T3 and T4 treatment on synaptic vesicle recycling in cerebellar granule neurons.

Figures 11A and 11B are graphs illustrating the rate analysis of the release of FMI-43-loaded synaptic vesicles from cerebellar granule neurons under continuous stimulus. Figure 11A illustrates the results of experiments in cultured cerebellar granule neurons. 10 Figure 11B illustrates the results of experiments in cultured cerebellar granule neurons expressing mutant myo5a (mutant myosin V).

### DETAILED DESCRIPTION

15 The present invention relates to the non-nuclear effects of thyroid hormone involving the truncated thyroid hormone receptors  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$ . In particular, the invention relates to methods of identifying compounds that bind to  $\Delta\text{TR}\alpha 2$  and are therefore useful, e.g., for treating disorders involving endocytosis, especially disorders of the nervous system and psychiatric disorders, for example, depression. The invention also involves methods of making transgenic animals and transgenic animals with altered  $\Delta\text{TR}\alpha 1$  and/or  $\Delta\text{TR}\alpha 2$ . Such 20 animals are useful, e.g., for identifying components of biochemical pathways involving  $\Delta\text{TR}\alpha 1$  and/or  $\Delta\text{TR}\alpha 2$ , thus providing additional targets for drug discovery. The transgenic animals of the invention are also useful for testing  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  ligands for effects unrelated to receptor binding.

25 Synaptic vesicle recycling is a fundamental process that is central to continued synaptic transmission. Movement of recycling synaptic vesicles between the reserve and readily releasable pools in the nerve terminal is the principal means by which the neuron replenishes spent vesicles, removes defective vesicles, regulates the quantity of neurotransmitter available for release and insures maximal refilling of the recycling synaptic vesicles. The present invention relates to the discovery that T4 and its analogs are involved 30 in the regulation of synaptic vesicle recycling through specific non-nuclear receptors, and that myosin V is a key molecular motor involved in this process. Thus, potential sites for pharmacological manipulation of synaptic vesicle availability are identified and can be used to identify compounds that modulate synaptic transmission.

5 Intron 7 of the thyroid hormone receptor gene contains an alternative transcriptional start site. The gene products of the alternative transcription are the truncated versions of the TR $\alpha$ s ( $\Delta$ TR $\alpha$ s).  $\Delta$ TR $\alpha$ 1 is encoded by exons 8-9 resulting in an approximately 16 kd protein.  $\Delta$ TR $\alpha$ 2 is encoded by exons 8-10 resulting in an approximately 25 kd protein. The invention is based, in part, on the discovery that these truncated receptor forms specifically  
10 bind to T4 and rT3 (Example 2). Nucleic acid sequences that encode  $\Delta$ TR $\alpha$ 1 and  $\Delta$ TR $\alpha$ 2 are known for some species, e.g., Genbank No. X07409 (rat) and Genbank No. X07751 (mouse), respectively.

In experiments designed to identify molecules that are associated with a  $\Delta$ TR $\alpha$ 1 or  $\Delta$ TR $\alpha$ 2,  $\Delta$ TR $\alpha$ 2 was discovered to be associated with synaptic vesicles and to mediate  
15 thyroid hormone-dependent endocytosis via interactions with myosin V (Examples 2-4). This finding is important because it suggests that  $\Delta$ TR $\alpha$ 2 is associated with the regulation of endocytosis, e.g., re-uptake of neurotransmitters.

Based on the discoveries disclosed herein, it is shown that T4 can mediate endocytosis and vesicle transport and is thus involved in neurotransmitter re-uptake. In the  
20 absence of T4, there can be decreased re-uptake. Thus, T4, rT3, and analogs of these hormones may be useful for treating disorders associated with the nervous system, for example, modulation of neurotransmitter re-uptake. The invention therefore encompasses methods of identifying compounds that affect the thyroid hormone-mediated vesicle transport system (Example 6).

25 The invention also features transgenic animals that lack expression of a  $\Delta$ TR $\alpha$  (Example 5), which are useful for identifying additional components of the vesicle transport system, providing additional novel drug targets.

#### 30 Screening Assays for Compounds That Bind to or Modulate a $\Delta$ TR $\alpha$ 1 or a $\Delta$ TR $\alpha$ 2 Polypeptide

One approach to identifying compounds that bind to a  $\Delta$ TR $\alpha$ 1 or a  $\Delta$ TR $\alpha$ 2 is to assay for compounds that interfere with the binding of a  $\Delta$ TR $\alpha$ 1 or a  $\Delta$ TR $\alpha$ 2 to a known binding partner (e.g., T4 or rT3). In one such method, interaction of a  $\Delta$ TR $\alpha$ 1 or  $\Delta$ TR $\alpha$ 2 with a

5 ligand is monitored using methods described herein or by other methods known in the art. Ligands that interfere with such binding are candidate compounds.

### *Screening assays*

10 The invention provides methods (also referred to herein as "screening assays") for identifying modulators, i.e., candidate or test compounds or agents (e.g., peptides, peptidomimetics, small organic or inorganic molecules, carbohydrates, aptamers, or other drugs) that bind to a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  polypeptide or have a stimulatory or inhibitory effect on, for example, expression or activity of a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  polypeptide. In some cases it may be desirable to use more than one of the assays to confirm the effect of a  
15 compound, e.g., if a molecule is identified as a candidate compound using one assay, a second, different assay may be employed to confirm the efficacy of the candidate compound's potential for influencing vesicle transport.

20 In one embodiment, an assay is a cell-based assay in which a cell that expresses a synaptic vesicle-associated form of a  $\Delta\text{TR}\alpha 2$  or a biologically active portion thereof, is contacted with a test compound and the ability of the test compound to bind to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  determined. For example, the cell can be of animal, e.g., mammalian, origin. Determining the ability of the test compound to bind to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  can be accomplished, for example, by coupling the test compound with a radioisotope or enzymatic label such that binding of the test compound to the polypeptide or biologically active portion  
25 thereof can be determined by detecting the labeled compound in a complex. For example, test compounds can be labeled with  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^3\text{H}$ , either directly or indirectly, and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively, test compounds can be enzymatically labeled, for example with, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by  
30 determination of conversion of an appropriate substrate to product. In another embodiment, the assay comprises contacting a cell which expresses a vesicle-associated form of a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  of the invention, or a biologically active portion thereof, on the cell surface with a known compound which binds to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test

5 compound to interact with the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ , by monitoring the ability of the test compound to preferentially bind to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  or a biologically active portion thereof as compared to the known compound.

In another embodiment, an assay is a cell-based assay comprising contacting a cell expressing a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ , or a biologically active portion thereof, with a test  
10 compound and determining the ability of the test compound to modulate (e.g., stimulate or inhibit) the activity of the polypeptide or biologically active portion thereof. The ability of the test compound to modulate the activity of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  or a biologically active portion thereof can be determined, for example, by confocal microscopy to assay alterations in synaptic vesicle transport within the cell in the presence of the test molecule compared to  
15 in the absence of the test molecule or in the presence of T4 or rT3.

The ability of a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  to bind to or interact with a target molecule can be determined by one of the methods described herein for determining direct binding. As used herein, a "target molecule" is a molecule with which a selected  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  binds to or interacts with in nature, for example, a T4 molecule, an F-actin, or other component of  
20 the vesicle recycling pathway (including structural components of a vesicle, e.g., a synaptic vesicle) with which a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  interacts in nature. The ability of a polypeptide of the invention to bind to or interact with a target molecule can be determined by monitoring the activity of the target molecule. For example, the activity of the target molecule can be determined by detecting an alteration in the activity of D2 (e.g., a decrease in D2 activity or  
25 expression in the presence of a T4 mimetic) or some other cellular response that is associated with binding to a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$ .

A cell-free assay can also be used to identify candidate compounds. In such an assay, a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  or biologically active portion thereof, is contacted with a test compound and the ability of the test compound to bind to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ , or  
30 biologically active portion thereof is determined. Binding of the test compound to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  can be determined either directly or indirectly as described herein. In one embodiment, the assay includes contacting the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  polypeptide or biologically active portion thereof, with a known compound that binds to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  to form an assay mixture, contacting the assay mixture with a test compound, and determining the

5 ability of the test compound to interact with the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ , wherein determining the ability of the test compound to interact with the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  comprises determining the ability of the test compound to preferentially bind to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  or biologically active portion thereof as compared to the known compound.

Another type of cell-free assay that can be used to identify candidate compounds  
10 comprises contacting a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  polypeptide, or biologically active portion thereof with a test compound and the ability of the test compound to modulate (e.g., stimulate or inhibit) the activity of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  or biologically active portion thereof is determined. The ability of the test compound to modulate the activity of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  can be determined, for example, by measuring the ability of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  to  
15 bind to a target molecule by one of the methods described herein for determining direct binding. In an alternative assay, the ability of the test compound to modulate D2 activity can be determined.

A cell-free assay can be used for identifying a candidate compound in which a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  polypeptide, or biologically active portion thereof, is contacted with a  
20 known compound that binds the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  to form an assay mixture. The assay mixture is then contacted with a test compound, and the ability of the test compound to interact with the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  (e.g., to displace the binding of the known compound) is determined. The ability of the test compound to interact with  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  indicates ability of the test compound to specifically bind to or modulate the activity of a target  
25 molecule.

In some of the cell-free assay methods of the present invention, it may be desirable to immobilize either the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  or the target molecule components of the assay to facilitate separation of complexed from uncomplexed forms of one or both of the components, as well as to accommodate automation of the assay. Binding of a test  
30 compound to the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ , or interaction of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  with a target molecule in the presence and absence of a candidate compound, can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. For this type of assay, a fusion protein can be provided that adds a domain that allows one or both of the proteins to be bound to a matrix.

5 For example, glutathione-S-transferase (GST) fusion proteins or glutathione-S-transferase  
fusion proteins can be adsorbed onto glutathione Sepharose beads (Sigma Chemical; St.  
Louis, MO) or glutathione-derivatized microtitre plates, which are then combined with the  
test compound or the test compound and either the non-adsorbed target molecule or a  $\Delta\text{TR}\alpha 1$   
or  $\Delta\text{TR}\alpha 2$ , and the mixture incubated under conditions conducive to complex formation  
10 (e.g., at physiological conditions for salt and pH).

Following incubation, the beads or microtitre plate wells are washed to remove any  
unbound components and complex formation is measured either directly or indirectly, for  
example, as described above. Alternatively, the complexes can be dissociated from the  
matrix, and the level of binding or activity of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  can be determined using  
15 standard techniques.

Other techniques for immobilizing proteins on matrices can also be used in the  
screening assays of the invention. For example, a  $\Delta\text{TR}\alpha 1$ ,  $\Delta\text{TR}\alpha 2$ , or target molecule can be  
immobilized utilizing conjugation of biotin and streptavidin. Biotinylated  $\Delta\text{TR}\alpha 1$ ,  
 $\Delta\text{TR}\alpha 2$ , or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide)  
20 using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals; Rockford,  
IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).  
Alternatively, antibodies reactive with a  $\Delta\text{TR}\alpha 1$ ,  $\Delta\text{TR}\alpha 2$  or a target molecule, but which do  
not interfere with binding of the  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  to a target molecule, can be derivatized to  
the wells of the plate, and unbound target or receptor ( $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ ) trapped in the  
25 wells by antibody conjugation. Methods for detecting such complexes, in addition to those  
described above for the GST-immobilized complexes, include immunodetection of  
complexes using antibodies reactive with a  $\Delta\text{TR}\alpha 1$ ,  $\Delta\text{TR}\alpha 2$ , or target molecule, as well as  
enzyme-linked assays which rely on detecting an enzymatic activity associated with the  
detecting antibody bound to  $\Delta\text{TR}\alpha 1$ ,  $\Delta\text{TR}\alpha 2$ , or target molecule.

30 Modulators of expression of a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  polypeptide are identified in a  
method in which a cell is contacted with a candidate compound and the expression of the  
selected mRNA or protein (i.e., the mRNA or protein corresponding to a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ ,  
or a nucleic acid encoding a  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$ ) in the cell is determined. The level of  
expression of the selected mRNA or protein in the presence of the candidate compound is



5 compared to the level of expression of the selected mRNA or protein in the absence of the  
candidate compound. The candidate compound can then be identified as a modulator of  
expression of a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  based on this comparison. For example, when  
expression of the selected mRNA or protein is greater (e.g., two-fold greater) in the presence  
of the candidate compound than in its absence, the candidate compound is identified as a  
10 stimulator of the selected mRNA or protein expression. Alternatively, when expression of  
the selected mRNA or protein is less (e.g., two-fold less) in the presence of the candidate  
compound than in its absence, the candidate compound is identified as an inhibitor of the  
selected mRNA or protein expression. Statistically significant differences (e.g.,  $p \leq 0.05$ )  
may be used as a means of determining whether there are significant differences in  
15 expression sufficient to indicate that a compound stimulates or inhibits expression. The level  
of the selected mRNA or protein expression in the cells can be determined by methods  
described herein.

#### *D2 as a marker in screening assays*

20 The enzyme type II 5' deiodinase (D2) is a particularly useful marker for expression  
or activity of a molecule that affects  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  expression or activity as described  
herein. This is because D2 activity rises in the presence of T4 and therefore will rise in the  
presence of molecules that mimic the effects of T4 (e.g., rT3). Thus, D2 activity can be  
assayed in an appropriate system to determine whether a molecule has T4-like activity, e.g.,  
25 is a candidate compound. D2 is especially useful as a marker of T4 or molecules with T4-  
like activity in *in vivo* assays. Such assays are known in the art and are described herein.

In an example of such an assay, a biological sample is sonicated in 50 mM HEPES  
(pH 7.0) containing 1 mM EDTA and 10 mM dithiothreitol (DTT). D2 activity is determined  
in the presence of 20 mM dithiothreitol and 1 mM 6-n-propylthiouracil. The ability of a test  
30 compound to modulate D2 expression or activity is compared to that of a known compound,  
for example, 2 nM rT<sub>3</sub> (Visser et al, 1982, Proc. Nat. Acad. Sci. USA 79:5080-5084).

5      *Test/candidate compounds*

In one embodiment, the invention provides assays for screening test compounds to see if they bind to or modulate the activity of a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  or biologically active portion thereof and so are candidate compounds. The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the "one-bead one-compound" library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer, or small molecule libraries of compounds (Lam, 1997, *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al., 1993, *Proc. Natl. Acad. Sci. USA* 90:6909; Erb et al., 1994, *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al., 1994, *J. Med. Chem.* 37:2678; Cho et al., 1993, *Science* 261:1303; Carrell et al., 1994, *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell et al., 1994, *Angew. Chem. Int. Ed. Engl.* 33:2061; and Gallop et al., 1994, *J. Med. Chem.* 37:1233. Molecular libraries are also available commercially from various sources.

Libraries of compounds may be presented in solution (e.g., Houghten, 1992, *Bio/Techniques* 13:412-421), or on beads (Lam, 1991, *Nature* 354:82-84), chips (Fodor, 1993, *Nature* 364:555-556), bacteria (U.S. Patent No. 5,223,409), spores (Patent Nos. 5,571,698; 5,403,484; and 5,223,409), plasmids (Cull et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:1865-1869), or phage (Scott and Smith, 1990, *Science* 249:386-390; Devlin, 1990, *Science* 249:404-406; Cwirla et al., 1990, *Proc. Natl. Acad. Sci. USA* 87:6378-6382; and Felici, 1991, *J. Mol. Biol.* 222:301-310).

Candidate compounds that are especially useful in the invention include reverse T3 (rT3), T4, T4 analogs, and flavones (Auf'mkolk et al., 1986, *J. Biol. Chem.* 261:11623-30; Auf'mkolk et al., 1986, *Biochem. Pharmacol.* 35:2221-2227; Auf'mkolk et al., 1984, *Hormone Metab. Res.* 16:188-92; Cody et al., 1986, *Prog. Clin. Biol. Res.* 213:373-82; Koehrle et al., 1986, *J. Biol. Chem.* 261:11613-22; Koehrle et al., 1986, *Prog. Clin. Biol. Res.* 213:359-71) including aurones, phloretins, and isoflavones (including phytoestrogens).

5 For example, the flavone EMD21388 and derivatives of EMD21388 are candidate compounds because the presence of EMD21388 can inhibit T4 deiodinases and can competitively block the binding of T4 to its serum binding partner, transthyretin. In astrocytes, EMD21388 decreases the quantity of D2 activity as does T4. Because the invention is based on the discovery of the association between myosin V and  $\Delta\text{TR}\alpha 2$ ,  
10 flavone derivatives are likely to be potent ligands and effectors of the actin-based endocytotic pathway. For example, potential derivatives (e.g., candidate compounds) for this class of compounds consist of the flavone backbone and up to 5 substituents (Figure 1). Figure 1 shows a flavinoid structure. For EMD21388,  $R_1$  and  $R_3$  are Br;  $R_2$  and  $R_5$  are  $-\text{OH}$ , and  $R_4$  is  $-\text{CH}_3$ . Combinatorial chemists can modify this backbone to achieve desired properties.  
15 Some candidate compound flavone-derived structures based on R-group substitutions are indicated in Figure 1.

Aurones are a class of flavones that are candidate compounds (Auf'mkolk et al., 1986, J. Biol. Chem. 261:11623-11630; Auf'mkolk et al., 1986, Biochem. Pharmacol. 35:2221-2227; Auf'mkolk et al., 1984, Hormone Metabol. Res. 16:188-192; Cody et al.,  
20 1986, Prog. Clin. Biol. Res. 213:373-382; Koehrle et al., 1986, J. Biol. Chem. 261:11613-11622; Koehrle et al., 1986, Prog. Clin. Biol. Res. 213:359-371) because they may function by inhibiting the deiodination of T4 by type I iodothyronine 5'-deiodinase. Figure 2 shows the basic structure for aurones. For certain aurones, it has been shown that if  $R_1$  (Figure 2) is either a halide (e.g., I),  $-\text{OH}$ , or  $-\text{H}$ , enzyme inhibition is favored. If the  $-\text{OH}$  is eliminated on  
25 C4, inhibition is weakened. Similarly if the 4'  $-\text{OH}$  is further substituted the molecule is a poor inhibitor.

A third class of potential candidate compounds that bind to  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  are analogs of thyroid hormone. Figure 3 depicts the basic structure for these compounds. Iodothyronine is the parent compound. Based on the effects of alanine side chain  
30 modifications that either enhance or diminish the ability of T4 or N-blocked T4 molecules to inactivate D2, the following criteria can be used to guide selection of test compounds for use in the invention:

1. Net negative charge on the alanine side chain impairs ligand binding to  $\Delta\text{TR}\alpha 2$ .

- 5           2.       Charge masking or elimination of the carboxyl group of the alanine side chain enhances ligand binding to  $\Delta\text{TR}\alpha 2$ .
3.       Elimination of one inner-ring iodine (3,5 positions) has no effect on ligand binding to  $\Delta\text{TR}\alpha 2$ . Elimination of two inner ring iodine may have no effect on ligand binding.
- 10       4.       Substitution of other bulky substitutions at the (3', 5' positions) does not generally affected binding to  $\Delta\text{TR}\alpha 2$ .

T4 is the compound illustrated by Figure 3 when  $R_1$ ,  $R_2$ ,  $R_3$ , and  $R_4$  are I, and  $R_5$  and  $R_6$  are protons. T3 exchanges -H for -I at  $R_2$  (or  $R_1$ ), rT3 exchanges a -H for -I at  $R_3$  (or  $R_4$ ); 3', 5'- T2 exchanges -H for -I at both  $R_3$  and  $R_4$ . Substitutions at  $R_6$  decrease the ability of the iodothyronine to cause D2 inactivation (i.e., bind to  $\Delta\text{TR}\alpha 2$ ) if  $R_5$  is a proton. However, if  $R_6$  is substituted, elimination of charge by masking at  $R_5$  will enhance the ability of the thyroid hormone analog to inactivate D2 (bind to  $\Delta\text{TR}\alpha 2$ ; Safran et al., 1993, J. Biol. Chem. 268: 14224-14229). All of these modified thyroid hormones have little, if any, thyromimetic effect in the nucleus; they are generally thought to be metabolically inactive.

Another flavone, phloretin, a 7,8-dihydrochaleone from plants, is a potent inhibitor of deiodination (Auf'mkolk et al., 1986, J. boil. Chem. 261:11623-11630; Auf'mkolk et al., 1986, Biochem. Pharmacol. 35:2221-2227; Auf'mkolk et al., 1984, Hormone Metab. Res. 16:188-192; Cody et al., 1986, Prog. Clin. Biol. Res. 213:373-382; Koehrle et al., 1986, J. Biol. Chem. 261: 11613-11622; Koehrle et al., 1986, Prog. Clin. Biol. Res. 213:359-371). Therefore phloretin and phloretin analogs are possible ligands for  $\Delta\text{TR}\alpha 2$  or  $\Delta\text{TR}\alpha 1$  and can be candidate compounds. The structure of this class of molecules is shown in Figure 4. In phloretin, R is a -OH.

#### *Cell types useful for assays*

The cells useful in the screening assays of the invention are generally from an animal. In most cases the cell will be from a mammal, e.g., a mouse or rat cell. Human cells, either primary, secondary, or cultured cells can also be used. Astrocytes are especially useful for the assays. Methods of culturing astrocytes are known in the art (e.g., Leonard, 1988, Biochem. Biophys. Res. Comm. 151:1164-1172), including methods for preparing and

5 culturing human astrocytes. Other cell types that are useful in the invention include neurons, mixed fetal rat brain cells, neuronal progenitor cells, embryonic stem cells, PC12 cells, C6 astrocytoma cells, and bone marrow stem cells.

This invention further pertains to novel agents identified by the above-described screening assays and uses thereof for treatments as described herein.

10

#### *Animal models*

Compounds identified as candidate compounds for therapeutic uses related to treating a disorder involving a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  protein can be tested in animal models of such disorders. For example, the learned helplessness model in rats can be used as a model for  
15 certain psychiatric disorders (e.g., Lucki, 1997. Behav. Pharmacol. 8: 523-532; Porsolt, 1979, Biomed. 30:139-140; Porsolt, 2000, Rev. Neurosci. 11:53-58).

#### Transgenic Animals

The invention encompasses the engineering of cells that can be used to produce  
20 nonhuman transgenic animals. For example, in one embodiment, such a cell is a fertilized oocyte or an embryonic stem cell that has been genetically engineered to lack competent promoters in intron 7 of a mouse thyroid hormone receptor gene. Another example of such a cell is one in which exon 10 of a mouse thyroid hormone receptor gene has been deleted or altered. Such engineered cells can be used to create non-human transgenic animals in which  
25 the modifications of the invention (i.e., modifications that alter the expression of a thyroid hormone receptor) have been introduced into their genome or homologously recombinant animals in which the endogenous thyroid hormone receptor gene has been altered. Such animals are useful for studying the function and/or activity of a  $\Delta\text{TR}\alpha 1$  or a  $\Delta\text{TR}\alpha 2$  protein, and for identifying and/or evaluating modulators of  $\Delta\text{TR}\alpha 1$  or  $\Delta\text{TR}\alpha 2$  activity.

30 A transgenic animal of the invention can be created by introducing nucleic acid containing the desired alteration in a thyroid hormone receptor gene into the male pronuclei of a fertilized oocyte, e.g., by microinjection or retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of

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5 expression of the transgene. If desired, a tissue-specific regulatory sequence(s) can be operably linked to the transgene to direct expression of the altered gene to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, are known in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, U.S. Patent No. 4,873,191 and in Hogan,  
10 Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the transgene in its genome and/or expression of mRNA encoding the transgene in tissues or cells of the animals. Additional methods of identifying such animals are described below in Example 4. A  
15 transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying the transgene can further be bred to other transgenic animals carrying other transgenes.

To create a homologously recombinant animal, a vector is prepared which contains at least a portion of a gene encoding a thyroid hormone receptor gene into which a deletion,  
20 addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the gene. In one embodiment, the vector is designed such that, upon homologous recombination, the endogenous gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "knock out" vector), e.g., by altering the promoter of intron 7 of a mouse thyroid hormone receptor gene. Alternatively, the vector can be designed such that, upon  
25 homologous recombination, the endogenous gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous protein). In the homologous recombination vector, the altered portion of the gene is flanked at its 5' and 3' ends by additional nucleic acid of the gene to allow for homologous recombination to occur between the exogenous gene carried  
30 by the vector and an endogenous gene in an embryonic stem cell. The additional flanking nucleic acid sequences are of sufficient length for successful homologous recombination with the endogenous gene. Typically, several kilobases of flanking DNA (both at the 5' and 3' ends) are included in the vector (see, e.g., Thomas and Capecchi, 1987, Cell 51:503 for a description of homologous recombination vectors). The vector is introduced into an  
35 embryonic stem cell line (e.g., by electroporation) and cells in which the introduced gene has

5 homologously recombined with the endogenous gene are selected (see, e.g., Li et al. (1992, Cell 69:915).

The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see, e.g., Bradley in Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, Robertson, ed., (IRL, Oxford, 1987) pp. 113-152). A chimeric  
10 embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA by germline transmission of the transgene. Methods for constructing homologous recombination vectors and homologous recombinant animals are  
15 described further in Bradley (1991) Current Opinion in Bio/Technology 2:823-829 and in PCT Publication Nos. WO 90/11354, WO 91/01140, WO 92/0968, and WO 93/04169.

In another embodiment, transgenic non-human animals can be produced which contain selected systems that allow for regulated expression of the transgene. One example of such a system is the cre/loxP recombinase system of bacteriophage P1. For a description  
20 of the cre/loxP recombinase system, see, e.g., Lakso et al. (1992) Proc. Natl. Acad. Sci. USA 89:6232-6236. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al., 1991, Science 251:1351-1355). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such  
25 animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase.

Clones of the non-human transgenic animals described herein can also be produced according to the methods described in Wilmut et al. (1997) Nature 385:810-813 and PCT  
30 Publication No's. WO 97/07668 and WO 97/07669.

The invention also includes populations of cells derived from transgenic animals of the invention.

These transgenic animals are useful for examining the role of  $\Delta TR\alpha$  polypeptides or proteins in whole animal physiology since the physiology and phenotypes of transgenic  
35 animals lacking a  $\Delta TR\alpha$  will reveal other systems besides nervous system where these

5 molecules may have a function. Such animals can also be used to study the role of  $\Delta TR\alpha s$   
in the nervous system. Transgenic animals of the invention are also useful, e.g., for studying  
non-TR-related effects of candidate compounds. This can be done by administering a  
candidate compound to a transgenic animal that does not express a  $\Delta TR\alpha$ . Effects of the  
candidate compound on the animal are examined. Adverse effects on the transgenic animal  
10 may be predictive of adverse effects that could occur should the candidate compound be used  
as a treatment for one of the disorders described herein. Candidate compounds with adverse  
effects are less likely to be useful as drugs to treat a disorder. The method of testing  
candidate compounds on transgenic mice lacking a  $\Delta TR\alpha$  can thus be used as a step in  
selecting those candidate compounds which are the best candidates to use for clinical studies  
15 in, e.g., humans.

#### Methods of Treatment

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20 The present invention provides for both prophylactic and therapeutic methods of  
treating a subject at risk of, or susceptible to, a disorder, or having a disorder, associated with  
expression or activity of a  $\Delta TR\alpha 1$  or a  $\Delta TR\alpha 2$ . Such expression or activity can be aberrant  
or normal. For example, disorders characterized by aberrant expression or activity of a  
 $\Delta TR\alpha 1$  or a  $\Delta TR\alpha 2$  protein or in which an alteration of normal expression or activity include  
certain psychiatric or neurologic disorders of the nervous system, e.g., mood disorders.  
Disorders that can be treated using the molecules of the invention (e.g., modulators of  
25  $\Delta TR\alpha 1$  or  $\Delta TR\alpha 2$  expression or activity) include bipolar disorder, major depression,  
attention deficit disorder, attention deficit hyperactivity disorder, and obsessive-compulsive  
disorder, as well as other disorders described herein.

In some cases, the therapeutic molecules identified using the methods described  
herein may be used as prophylactic treatments of those considered at-risk for a disorder that  
30 is treated using the therapeutic molecule.



5            *Effective Dose*

Toxicity and therapeutic efficacy of the compounds of the invention can be determined by standard pharmaceutical procedures, using either cells in culture or experimental animals to determine the LD<sub>50</sub> (the dose lethal to 50% of the population) and the ED<sub>50</sub> (the dose therapeutically effective in 50% of the population). The dose ratio  
10 between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD<sub>50</sub>/ED<sub>50</sub>. Compounds that exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue to minimize potential damage to non-target cells and, thereby, reduce side effects.

15            The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED<sub>50</sub> with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods of the  
20 invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can also be calculated in animal models to achieve a circulating plasma concentration range that includes the IC<sub>50</sub> (that is, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in  
25 plasma can be measured, for example, by high performance liquid chromatography. Dosages are from about 1 µg to 500 mg per day. Typically, dosages are from about 5 µg – 500 µg per day. Because circulating plasma levels of a compound can differ substantially from levels circulating in the cerebrospinal fluid (CSF), in some cases it may be preferable to measure CSF levels of the compound.

30            Formulations and Use

Pharmaceutical compositions for use in accordance with the present invention can be formulated in a conventional manner using one or more physiologically acceptable carriers or excipients.

35            Thus, the compounds and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically

5 acceptable excipients such as binding agents (for example, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (for example, lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (for example, magnesium stearate, talc or silica); disintegrants (for example, potato starch or sodium starch glycolate); or wetting agents (for example, sodium lauryl sulphate). The tablets may be  
10 coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (for example, sorbitol syrup, cellulose derivatives or  
15 hydrogenated edible fats); emulsifying agents (for example, lecithin or acacia); non-aqueous vehicles (for example, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (for example, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration may be suitably formulated to give  
20 controlled release of the active compound. For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, for example,  
25 dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

30 The compounds may be formulated for parenteral administration by injection, for example, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, for example, in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as  
35 suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, for example, sterile pyrogen-free water, before use.

5 The compounds may also be formulated in rectally administered compositions such as suppositories or retention enemas, for example, containing conventional suppository bases such as cocoa butter or other glycerides.

10 In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

15 The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

20 The therapeutic compositions of the invention can also contain a carrier or excipient, many of which are known to skilled artisans. Excipients which can be used include buffers (for example, citrate buffer, phosphate buffer, acetate buffer, and bicarbonate buffer), amino acids, urea, alcohols, ascorbic acid, phospholipids, proteins (for example, serum albumin), EDTA, sodium chloride, liposomes, mannitol, sorbitol, and glycerol. The compounds of the invention can be administered by any standard route of administration. For example, administration can be parenteral, intravenous, subcutaneous, intramuscular, intracranial, 25 intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, transmucosal, or oral. The compound can be formulated in various ways, according to the corresponding route of administration. For example, liquid solutions can be made for ingestion or injection; gels or powders can be made for ingestion, inhalation, or topical application. Methods for making such formulations are well known and can be found 30 in, for example, "Remington's Pharmaceutical Sciences."

### Uses

35 The screening methods and transgenic animals of the present invention are useful for identifying compounds that can affect endocytosis and vesicle transport. The invention is especially useful for identifying compounds that affect synaptic vesicle transport and as such can be used for treating disorders involving the nervous system, e.g., antidepressants. Without committing to any particular theory, the data upon which the invention is based suggest that thyroid hormone can target vesicle pools. Neurotransmitters are released from axons and many are recycled back into the cell through re-uptake mechanisms involving

5 vesicle pools. There are differences between vesicles within the pools, e.g., vesicles located near the axonal hillock tend to contain greater concentrations of neurotransmitters than do those located near the axon terminal. Because the data upon which the present invention is based suggest that thyroid hormone is likely to cause vesicles to move further from the nerve terminus, the vesicles are likely to contain greater concentrations of neurotransmitter, causing  
10 less depletion of neurotransmitters. Some anti-depressants act by raising the amount of a specific neurotransmitter (e.g., serotonin) in the synaptic cleft. The modulators of the present invention may therefore act to promote more efficient recycling of neurotransmitters between the cleft and the cell. Thus, for example, lower concentrations of anti-depressants may be required to achieve a therapeutic effect, and/or there may be improved therapeutic effect of  
15 an anti-depressant delivered at standard dosages.

### EXAMPLES

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

#### Example 1: Materials and General Methods

##### *Materials*

T4 (thyroxine), Triton X-100<sup>®</sup>, ATP, bt2cAMP, hydrocortisone, colchicine, bovine serum albumin, and rabbit anti-actin IgG were obtained from Sigma (St. Louis, MO).  
25 Dulbecco's modified Eagle's medium, antibiotics, Hank's solution, and trypsin were purchased from GIBCO (Grand Island, NY). Acrylamide was purchased from National Diagnostics (Atlanta, GA). TEMED and ammonium persulfate was purchased from Bio-Rad (Richmond, CA). Hybond ECL nitrocellulose was obtained from Amersham (Arlington Heights, IL); horseradish peroxidase conjugated goat, anti-rabbit IgG was obtained from  
30 Promega (Madison, WI); rabbit anti-GFP IgG was from Clontech (Palo Alto, CA). The Lumiglo<sup>®</sup> chemiluminescent detection system was obtained from Kirkegaard and Perry (Gaithersburg, MD). BrAc[<sup>125</sup>I]T4 was synthesized as described in Köhrle et al. (1990, J. Biol. Chem. 265:6155-6163). Restriction endonucleases and DNA modifying enzymes were purchased from New England Biolabs (Beverly, MA).

### *Culture Preparation*

Astrocytes were prepared from one day old neonatal rats as described in Leonard (1988, Biochem. Biophys. Res. Comm. 151:1164-1172) and cultured in Growth medium composed of Dulbecco's modified Eagle's medium supplemented with 10% supplemented bovine calf serum, 50 units/ml penicillin, 90 units/ml streptomycin. Cells were grown to confluence in 75 cm<sup>2</sup> culture flasks in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37° C, and used at passages 1-3.

### *Myosin V*

The Genbank number of the myosin V (also referred to as myosin 5a; myo 5a) sequence used for the experiments described below is X57377.

### *Antibody Preparation*

Synthetic peptides corresponding to the last 22 amino acids C-terminus of myosin V (NH<sub>2</sub>-YSLALETIQIPASLGLGFIARV-COOH; SEQ ID NO:1) were synthesized. An N-terminal tyrosine was added to facilitate diaminobenzidine coupling to KLH (Keyhole Limpet hemocyanin) and for radioiodination. Peptide-KLH conjugate (750 µg KLH conjugate/500 µl) was mixed with an equal volume of Complete Freund's adjuvant and injected intradermally at 20 sites on the backs of 2.2 kg female New Zealand White Rabbits.

Antibodies were also raised against an internal myosin V domain corresponding to the last IQ domain and the coiled-coil region (residues 892 to 1040, myosin V<sup>cc</sup>). PCR amplified myosin V cDNA was prepared using site specific, 20-mer oligonucleotides; the approximately 500 bp fragment was cloned into the EcoRV site of the pThioHis B prokaryotic expression vector (Invitrogen, San Diego, CA) and the fusion protein was synthesized in IPTG induced *E. coli*. The myosin V<sup>cc</sup> fusion protein was purified on Ni-Sepharose (Clontech) from cell lysates according to manufacturer's instructions. Approximately 75 µg of myosin V<sup>cc</sup> was diluted 1:1 with Freund's complete adjuvant and used to immunize rabbits as described above.

The specificities of the rabbit anti-myosin V antisera were documented by

5 immunoblot analysis. Brain homogenates were prepared from phenotypically normal, heterozygous (myosin V <sup>+/</sup>-) and myosin V-deficient, homozygous *dilute* mouse (myosin V <sup>-</sup>-). Both antibodies recognized a 190 kDa protein in the brain homogenates containing myosin V (heterozygotes) but showed no immunoreactive band in the homogenates of *dilute* mouse brain that lacks myosin V.

10

### *Immunoblotting*

Total cell protein was measured by the Bradford dye binding assay (Sigma, St. Louis, MO). To prepare samples, 20-50 µg of cellular protein was reduced, denatured and separated by SDS-PAGE (Laemmli, 1970, Nature 227: 680-685). Resolved proteins were transferred to  
15 Hybond membranes by electrotransfer using a Semi-Dry transfer apparatus (200 mA for 1 hour). The membrane was blocked in TRIS-buffered saline (pH 7.5) containing, 0.1% Tween 20 (v/v) and 5 % powdered milk (w/v; TTBS/5% milk). Immunoblots were then probed with primary antibodies (1: 500 for anti-Myosin V antisera; 2 µg/ml for anti-GFP (Green Fluorescent Protein IgG) for 16 hours at 4°C. After washing, immune complexes  
20 were detected with HRP (horseradish peroxidase)-conjugated, goat, anti rabbit IgG (1: 2000 final dilution) and the specific complexes visualized by chemiluminescence and Kodak XOMAT® AR5 radiographic film.

### *Construction of replication-deficient, myosin V viral vectors*

25 The 3280 base pair fragment containing the coding sequence of the globular myosin V tail cDNA (myosin V tail) were excised from clone D64 (Huang et al., 1998, Genetics 148:1963-1972) with *Ssp*I and *Eco*47III, and ligated into the *Eco*RV site of the AdpREC shuttle vector (T. Kowalik, UMMC). The shuttle construct was linearized with *Eco*RI and cotransfected with *Xba*-*Cla*I linearized Ad5-gal into HEK 293 cells (ATCC No. CRC-1573)  
30 using lipofectin according to manufacturer's instructions. Replication-deficient Ad5-myoV containing virus particles were purified from the HEK293 cell lysates by cesium chloride gradient centrifugation. Other suitable vectors may be used.

Expression of myosin V from Ad5-myoV infected cells was confirmed by Western blot analysis. The Ad5-p29 GFP virus particles were generated as described in Visser et al.

5 (1982, Proc. Nat. Acad. Sci. USA 79:5080-5084).

### *Immunocytochemistry*

Astrocytes were seeded onto to poly-d-lysine (10 µg/ml) coated coverslips.

Myosin V distribution was determined in bt2cAMP-stimulated astrocytes treated or untreated  
10 with T4. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton  
X-100®.

To visualize myosin V, anti-myosin V IgG (C-terminus) was used at a final dilution  
of 1: 500. The immune complexes were visualized using a Texas Red-conjugated donkey,  
anti-rabbit IgG (dil 1: 50). Twenty to 50 random fields were examined by fluorescence  
15 microscopy.

### Example 2: Specificity of $\Delta\text{TR}\alpha 1$ and $\Delta\text{TR}\alpha 2$ Binding

The properties of thyroid hormone binding to non-nuclear components of the cell  
were examined using  $\Delta\text{TR}\alpha 2$  (T4 Inactivating Protein; TIP)-containing, nuclei-free extracts  
20 of cultured rat astrocytes. Reverse T3 (rT3) was used as the ligand because of its potent  
biological effect on D2 (type II 5'deiodinase) endocytosis and its low avidity for any  
contaminating nuclear thyroid hormone receptors. Both rT3 and T4 specifically displace rT3  
with  $K_d$ 's of approximately 0.3-0.5 nM. Subcellular localization of the specific rT3 binding  
sites revealed that  $\Delta\text{TR}\alpha 2$  was most abundant in subcellular fractions containing cellular  
25 membranes and the cytoskeleton.

$\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  polypeptides were synthesized by cell-free translation of  
appropriate segments of the rat  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  cDNA using the coupled transcription-  
translation kit from Promega® (T<sub>N</sub>T) according to the manufacturer's instructions. Synthesis  
of the appropriate sized translation product(s) was confirmed by SDS-PAGE. The thyroid  
30 hormone displacement curves for rT3 binding to  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  demonstrated that both  
 $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  specifically bound from 3 to 5% of the total [<sup>125</sup>I]rT3. Also, both T4 and  
rT3 specifically displaced [<sup>125</sup>I]rT3 with  $K_d$ 's of about 0.3 to 1nM. These data are identical  
to those for native  $\Delta\text{TR}\alpha 2$  found in astrocyte lysates. T3 did not displace [<sup>125</sup>I]rT3 from  
either thyroid hormone receptor at concentrations up to 100 nM, consistent with the failure of

5 T3 to initiate actin-based endocytosis or bind to TIP in astrocyte lysate. Control studies done with cell-free translated  $\beta$ -galactosidase showed no specific rT3 binding.

These data indicate that  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  polypeptides can act as high affinity thyroid hormone binding proteins ( $K_d \sim 0.4$  nM) that favors fully outer ring substituted iodothyronines. Such a protein is present in rat astrocytes. These data also show that the  
10 thyroid hormone specificity of  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  is different from that of the nuclear thyroid hormone receptors. Both  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  show similar thyroid hormone binding preferences and affinities.

### Example 3: Identification of $\Delta\text{TR}\alpha$ 's in Myosin V-Enriched Synaptic Vesicles from Rat

#### 15 Brain

To further examine the role in the brain of the truncated thyroid hormone receptors ( $\Delta\text{TR}\alpha$ 's), myosin V-enriched synaptic vesicles were prepared from rat cerebral cortex and cerebellum using the method of Evans et al. (1998, J. Cell Sci. 111: 2055-2066), and characterized by immunoblot for the presence of the expected synaptic vesicle proteins and  
20 for  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$ .

Myosin V comprises approximately 0.3% of total protein in brain. Cultured astrocytes were examined in untreated (control) and T4-treated cells for the presence and distribution of myosin V using Western Blot analysis and immunocytochemistry. Cultured astrocytes were treated overnight with 10  $\mu\text{M}$  retinoic acid  $\pm 10$  nM T4. Cells were collected by scraping and  
25 lysed by two freeze-thaw cycles. Triton X-100<sup>®</sup> supernatants and pellets were prepared as described herein and equivalent volumes of resuspended Triton pellet and Triton supernatant, and whole cell lysate were separated on 5-20% linear gradient SDS-PAGE gels and transferred to nitrocellulose. Immunoblot analysis was then performed.

More than 80% of the 190 kDa, immunoreactive myosin V was found in the Triton-  
30 insoluble pellets prepared from retinoid-treated astrocytes in the absence or presence of T4. Pre-incubation of the anti-myosin V antibody with excess blocking peptide (10  $\mu\text{g}/\text{ml}$ ) completely eliminated the 190 kDa immunoreactive band, indicating that the myosin V present in astrocytes is predominantly associated with the F-actin cytoskeleton. No differences in total actin content were observed between the Triton supernatant and Triton



5 pellet from retinoid-treated astrocytes that were grown in absence and presence of thyroid hormone. More than 95% of the immunoreactive actin was found in the Triton-insoluble pellet in both thyroid hormone deficient and T4-treated cells. These data demonstrate that retinoid-treated, thyroid hormone deficient astrocytes contain a fully polymerized actin cytoskeleton and that myosin V is constitutively bound to F-actin.

10 Using immunocytochemistry to localize myosin V in astrocytes treated in the absence and presence of 10 nM T4, it was observed that immunoreactive myosin V was found in the perinuclear space, and diffusely distributed throughout the cell in the absence and in the presence T4. These data indicate that astrocytes express abundant myosin V and that quantity of myosin V in the cell is not dependent on thyroid hormone. Thus, it is not expected that  
15 candidate compounds of the invention will affect the amount of myosin V present in a cell.

Immunocytochemical methods were used to examine proteins associated with myosin V-enriched synaptic vesicles isolated from rat cerebellum and cerebral cortex. The synaptic vesicles were found to contain abundant immunoreactive myosin V, actin, and Rab3. Neither synaptic vesicle pool showed any TR $\alpha$ 1-derived protein(s) using antibodies that  
20 recognize the unique C-terminus of these polypeptides. By contrast, both synaptic vesicle preparations showed a TR $\alpha$ 2 signal with the expected Mr of  $\Delta$ TR $\alpha$ 2.

These data show that an immunoreactive ~26 kDa polypeptide ( $\Delta$ TR $\alpha$ 2) is present in synaptic vesicles enriched for myosin V and indicate that  $\Delta$ TR $\alpha$ 2 and its ligands play a role in synaptic vesicle function.

25 Example 4: Cellular and Subcellular Localization of the TR $\alpha$ -derived Polypeptides in Mixed Brain Cell Cultures and in Rat Astrocytes

The subcellular distribution of immunoreactive thyroid hormone receptor-derived proteins in primary brain cell cultures and in cultured astrocytes was examined. Primary  
30 brain cell cultures were fixed with 0.4% paraformaldehyde, permeabilized with Triton X-100®, and stained for TR $\alpha$ -derived proteins using specific antisera directed against the unique C-termini of TR $\alpha$ 1 and TR $\alpha$ 2 proteins, and visualized by staining with a secondary tagged antibody. Consistent with previous observations (Kolodny et al., 1985, Endocrinol.  
117: 1848-1857), immunoreactive TR $\alpha$ 1 proteins were found in the nuclei of astrocytes and

5 neuronal cells. However, TR $\alpha$ 2-derived proteins were not localized to the nucleus in either astrocytes or neurons, but appeared as donut shaped structures located throughout the astrocyte or as discrete punctate signals along neuronal fibers. This latter pattern is diagnostic for synapses in neuronal cultures (Wong et al., 1999, Neurosci. 89:221-233), and is consistent with the presence of  $\Delta$ TR $\alpha$ 2-derived protein in nerve terminals.

10 The subcellular distribution of immunoreactive TR $\alpha$ 2-derived proteins in cultured astrocytes was influenced by the presence of either T4 or rT3. In these experiments astrocytes were grown in serum-free medium, treated for 20 minutes with either rT4 or rT3, and fixed, permeabilized, and stained as described above. In the absence of thyroid hormone, TR $\alpha$ 2-derived proteins were distributed throughout the cell and along the cell periphery, but  
15 were not concentrated in the cell nucleus. Addition of either rT3 or T4 in 0.1% bovine serum albumin as a carrier led to the coalescence of the TR $\alpha$ 2-derived proteins around the perinuclear space of the astrocyte, a distribution similar to that shown for the TH-dependent endocytosis of p29 (D2) vesicles. Thus, it appears that TR $\alpha$ 2-derived protein(s) are found in the nerve terminals of neurons grown *in vitro* and these immunoreactive TR $\alpha$ 2-derived  
20 polypeptides show thyroid hormone-dependent internalization and relocalization in cultured astrocytes.

#### Example 5: Characterization of the TH-dependent Binding of Vesicle Docking Proteins and

##### $\Delta$ TR $\alpha$ 2 to F-actin Fibers through the C-terminus of Myosin V

25 Myosin V is a 190 kDa protein that is widely distributed in the brain (Cheney et al, 1993, Cell 75, 13-23; Wagner et al, 1992, J. Cell Biol. 119: 163-170). p29 (D2) vesicle pull-down (immunoprecipitation) assays or F-actin isolation (*supra*) revealed that Rab3, synaptophysin, and synaptotagmin were constitutive elements of p29 vesicles, thus demonstrating a link between p29 vesicles and synaptic vesicles. Both myosin V and  $\Delta$ TR $\alpha$ 2 showed thyroid hormone-dependent association with p29 vesicles. In contrast, when the  
30 proteins bound to the F-actin cytoskeleton were analyzed, both myosin V and  $\Delta$ TR $\alpha$ 2 appeared to be constitutively bound to F-actin, while the synaptic vesicle proteins showed thyroid hormone-dependent association with the microfilaments. These data add additional support to the discovery that thyroid hormone directly influences synaptic vesicle transport.

5            *Characterization of the interaction(s) between the myosin V tail and p29 vesicles.*

Since the approximately 80 kDa globular tail of myosin V specifically binds to synaptic vesicles (Prekeris and Terrian, 1997, J. Cell Biol 137:1589-1601), myosin V mutants lacking the actin-binding head would be expected to compete with the native, F-actin bound myosin V for the p29 vesicles, thereby blocking the thyroid hormone-dependent binding of p29 vesicles to F-actin. Initial studies used the entire C-terminus of myosin V synthesized *in vitro* from a 4.2 kb fragment (nt2911-nt7087) of the myosin V cDNA ( $\Delta$  myosin V tail ) using a coupled transcription and translation system (TNT<sup>®</sup>, Promega). Cell-free synthesis of the appropriate myosin V fragment was confirmed by immunoblot and an 88 kDa band was detected using anti-myosin V antibodies directed against the C-terminal 22 amino acids.

10            Increasing volumes (5 or 10  $\mu$ l) of  $\Delta$  myosin V tail or a comparable volume of control reticulocyte lysate were added to the *in vitro* actin binding assay (see *infra*) and pre-incubated for 20 minutes at 37°C. Actin binding of the p29 vesicles was then initiated by addition of 10 nM T4, and the Triton-insoluble (F-actin bound) pellet was separated from the Triton-soluble (vesicle) fraction.

20            Addition of 5  $\mu$ l of  $\Delta$  myosin V tail blocked about 50% of the T4-dependent binding of p29 vesicles to F-actin, while the addition of 10  $\mu$ l of  $\Delta$  myosin V tail blocked more than 95% of the p29 binding. In control binding assays, addition of up to 10  $\mu$ l of the reticulocyte lysate failed to affect the T4-dependent p29 vesicle binding to F-actin. These data indicate that the loss of the actin-binding head of myosin V generates a Triton-soluble myosin V mutant that will compete with the wild type motor and block the T4-dependent binding of p29 vesicles to the actin cytoskeleton and so demonstrates the involvement of myosin V in the T4-dependent binding. Thus,  $\Delta$ TR $\alpha$ 2 interacts with the C-terminal region of myosin V, based on the ability of a headless myosin V deletion mutant lacking the actin binding head, the neck and coiled-coil domains to bind  $\Delta$ TR $\alpha$ 2 and shift the distribution of this effector molecule to the cytoplasm.

25            30

Monitoring myosin V binding can therefore be used as an assay for T4 analogs involved in T4-dependent binding of p29 vesicles to the actin cytoskeleton.

*Use of p29 GFP labeled vesicles to analyze myosin V function in rat astrocytes*

5 To define the specific region(s) of the myosin V tail that interact with the p29 vesicle,  
a series of deletion mutations were created based on the dominant negative effect of  
exogenous myosin V tail on p29 vesicle binding. Competition analysis of the myosin V  
deletion mutations on T4-dependent binding of p29 vesicles to F-actin was performed using a  
modified *in vitro* assay in which a GFP-tagged p29 fusion protein (p29<sup>GFP</sup>) replaced the  
10 radioaffinity labeled native p29 (Farwell et al., 1990, J. Biol. Chem. 265:18546-18553;  
Farwell et al., 1993, J. Biol. Chem. 268:5055-5062). This allowed direct evaluation of the  
binding of fluorescent vesicles to F-actin without affinity radiolabeling of the p29 and  
subsequent SDS-PAGE analysis.

To confirm that the p29<sup>GFP</sup>-labeled vesicles showed the same hormone dependent  
15 attachment to the actin cytoskeleton as observed for the radioaffinity labeled p29 (Farwell et  
al., 1990, *supra*; Farwell et al., 1993, *supra*) exogenous p29<sup>GFP</sup> was introduced into the  
astrocytes used to prepare the *V-lysate* by infection with replication deficient Ad5-p29<sup>GFP</sup>  
virus particles. Equal volumes of *F-lysate* and *V-lysate* containing p29<sup>GFP</sup>-labeled vesicles  
were incubated with increasing concentrations of T4, rT3, or T3 (0-100 nM) for 20 minutes at  
20 37° C, and Triton-soluble and Triton-insoluble fractions were separated by microfuge  
centrifugation. Specific fluorescence at 510 nm (excitation, 488 nm) was then determined in  
the resuspended Triton-insoluble pellets. Dose-response studies demonstrated that both T4  
and rT3 showed concentration dependent increases in the quantity of p29<sup>GFP</sup> bound to the F-  
actin with EC50's of about 0.5 nM, identical to those reported previously (Farwell and  
25 Leonard, 1992, Endocrinol., 131:721-728). As expected, T3 did not increase p29<sup>GFP</sup> vesicle  
binding to F-actin, except for a modest 10-20% observed at 100 nM T3, the highest  
concentration of hormone used. These data illustrate that the modified *in vitro* binding assay  
faithfully reproduces the results obtained with affinity radiolabeled p29.

To determine if the myosin V tail could be captured by the p29<sup>GFP</sup> vesicle, an  
30 exogenous myosin V tail was introduced into astrocytes constitutively expressing p29<sup>GFP</sup> by  
infection with Ad5-ΔMyo V tail virus particles and examined the effects of T4 on the binding  
of the myosin V tail to immunopurified p29<sup>GFP</sup> vesicles. Astrocytes expressing p29<sup>GFP</sup> were  
grown in serum-free media and treated for 16 hours with 1 mM bt<sub>2</sub>cAMP and 100 nM

5 hydrocortisone. A separate pool of astrocytes was treated in serum-free media supplemented with 10  $\mu$ M retinoic acid. Cells were collected by scraping, and lysed by two freeze-thaw cycles. 100  $\mu$ g aliquots of *p29<sup>GFP</sup> V-lysate* and *F-lysate* were incubated, in triplicate, for 20 minutes with increasing concentrations of T4, T3, or rT3. Triton-soluble (vesicle) and Triton-insoluble (F-actin) fractions were separated by microfuge centrifugation. Triton pellets were  
10 resuspended in 300  $\mu$ l of PBS and fluorescence at 510 nm (excitation 488 nm) determined and relative fluorescence was reported as arbitrary units. Cells were treated with 10 nM T4 for twenty minutes and a Triton-soluble supernatant was prepared. Vesicles containing the *p29<sup>GFP</sup>* were immunoprecipitated by anti-GFP IgG (2  $\mu$ g/ml) and protein in the immunoprecipitates were resolved by SDS-PAGE. Immunoblot analysis of vesicle-associated myosin V was performed using the anti-myosin V antibody directed against the C-terminus.

In control cells expressing the *p29<sup>GFP</sup>* alone, no myosin V immunoreactive protein was detected in the purified vesicle pool since the native myosin V is constitutively bound to the Triton-insoluble F-actin (see above). In contrast, expression of the truncated  
20 ( $\Delta$ ) myosin V in *p29<sup>GFP</sup>* expressing cells showed a T4-dependent association between myosin V and *p29<sup>GFP</sup>* vesicle, as judged by the appearance of an 88 kDa immunoreactive band. Since there was no interaction between the myosin V tail and *p29* vesicle in the absence of T4, these data show that the direct interaction between the *p29* vesicle and myosin V is hormonally regulated.

25 Myosin V truncation mutants were used to further define the role of myosin V in mediating the binding of *p29<sup>GFP</sup>* to the F-actin cytoskeleton. Figure 5 shows a schematic diagram of the myosin V tail deletion mutations studied and the results of competition experiments investigating the amount of *p29* binding to myosin V in the presence of each of the truncation mutants.

30 To perform the competition experiments, the deletion mutants were synthesized by cell-free translation and the synthesis of the correct myosin V polypeptides was confirmed by Western blot analysis. The quantity of each mutant protein synthesized was determined by [<sup>35</sup>S]-methionine incorporation. Astrocytes expressing *p29<sup>GFP</sup>* were grown in serum-free media and treated for 16 hours with 1 mM bt2cAMP and 100 nM hydrocortisone. *p29<sup>GFP</sup> V-*

5 *lysate* and *F-lysate* were prepared as described herein. 100 µg aliquots of *p29<sup>GFP</sup> V-lysate*  
and *F-lysate* were pre-incubated, in triplicate, for 20 minutes at 37°C in the presence of  
individual Δ myosin V mutant proteins (~2-3 pmol polypeptide/50 µl mixture) were added to  
the actin-binding assay and pre-incubated for 20 minutes at 37°C. T4 (10 nM) was then  
added, the mixtures incubated for 20 minutes at 37° C, and F-actin bound, fluorescent  
10 *p29<sup>GFP</sup>* vesicles were then isolated in the Triton-insoluble pellet.

Addition of the Δ myosin V middle protein, corresponding to amino acids 504-1307,  
did not compete with native myosin V for the T4-dependent binding of *p29* to F-actin.  
Addition of the entire myosin V tail (residues 953 to 1852) decreased *p29* binding to F-actin  
by more than 75% ( $p < 0.01$ ). Addition of progressively shorter myosin V tail deletion  
15 mutants, Δ myosin <sup>1513</sup> (residues 1513-1852), Δ myosin <sup>1767</sup> (residues 1767-1852), and  
Δ myosin <sup>1830</sup> (residues 1830-1852), demonstrated that all competed with native myosin V  
and decreased T4-dependent *p29* binding by more than 75%.

Consistent with the idea that the C-terminus of myosin V contains the vesicle binding  
region, synthesis of a myosin V tail lacking the last 44 residues (residues 953-1803) yielded a  
20 truncation mutant that did not compete with native myosin V for T4-dependent *p29* vesicle  
binding. These data demonstrate that the vesicle binding region of myosin V that is  
responsible for T4-dependent, tethering of the *p29* containing vesicles is located in the last 22  
amino acids found at the C-terminus of myosin V. These data show that peptides containing  
at least the last 22 amino acids of the C-terminus of myosin V may be useful in assays of  
25 candidate compounds that affect T4-dependent binding to vesicles.

*Antibodies directed against the C-terminus of myosin V block the T4-dependent  
binding of p29 vesicles*

Antibody inhibition experiments were performed to confirm that ΔTRα2 mediates the  
30 TH-dependent binding of the *p29* vesicle to actin fiber and to confirm the location of the  
vesicle-tethering region of myosin V. Two antibodies were used; one directed against the  
coiled coil domain (residues 892 and 1040), and one directed against the C-terminal 22 amino  
acids (residues 1830-1852).

*V lysates* prepared from astrocytes over-expressing *p29<sup>GFP</sup>* were pre-incubated for

5 30 minutes at room temperature with antisera raised against  $\Delta\text{TR}\alpha 2$ ,  $\Delta\text{TR}\alpha 1$ , the C-terminus of MyoV (encoded by  $\text{MyoV}^{\text{tail}}$ , Figure 5), the coiled-coil region of MyoV (encoded by  $\text{MyoV}^{\text{coil-coil}}$ , Figure 5) and GFP (green fluorescent protein). T4 (10 nM) and actin fiber-containing *F-lysate* was then added, the mixtures incubated at 37°C for 20 minutes, and the Triton-insoluble fraction isolated by centrifugation. As shown in Figure 9, anti-GFP and anti-  
10  $\text{MyoV}^{\text{coil-coil}}$  antibodies had no effect on p29 vesicle binding. Anti- $\text{MyoV}^{\text{tail}}$  antibody showed concentration dependent inhibition of p29 binding. Anti- $\text{TR}\alpha 1$ -specific antibodies failed to alter p29 binding to actin fibers, while anti- $\text{TR}\alpha 2$  antibodies showed a concentration dependent inhibition of p29 binding similar to that of the anti- $\text{MyoV}^{\text{tail}}$  antibody.

The data indicate that antibodies directed against the extreme C-terminus of myosin V  
15 nearly completely blocked the T4-dependent binding of p29 vesicles, while antibodies directed against the coiled-coil domain generated only a modest 15-20% decrease in p29 vesicle binding. Control rabbit immunoglobulins had no effect of the T4-dependent binding of p29 vesicles to myosin V. These data confirm the assignment of the vesicle-tethering domain to the C-terminus of myosin.

20 These data indicate that the interaction between the p29 vesicle and F-actin is mediated by thyroid hormone-dependent binding of the vesicle to myosin V, presumably through  $\Delta\text{TR}\alpha 2$ . These data support the assertion that thyroid hormone plays a role in mediating actin-based synaptic vesicle endocytosis/transport, specifically through the binding of myosin V to p29 vesicles.

25 p29 is the substrate binding subunit of D2. A decrease in the amount of D2 expression or activity is therefore useful for modulating actin-based endocytosis, e.g., is a useful method of altering neurotransmitter reuptake.

#### Example 5: Transgenic Mice Lacking $\Delta\text{TR}\alpha 1$ and $\Delta\text{TR}\alpha 2$ Binding Activity

30 The invention also relates to transgenic animals with altered  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$  expression. The loss of either of these receptors is expected to result in animals with profound defects in neural integration, poor synaptic function, and premature death. These animals are useful for characterizing the role of  $\Delta\text{TR}\alpha 1$  and/or  $\Delta\text{TR}\alpha 2$  in neurogenesis and synapse function, providing information regarding the role of thyroid hormone in the brain,

- 5 identifying targets for use in drug discovery, and testing candidate compounds for their effects, including effects that are not mediated by  $\Delta\text{TR}\alpha 1$  and  $\Delta\text{TR}\alpha 2$ .

General methods of generating such animals are described above: the following provides specific examples of generating such transgenic animals.

10 *Cloning of exons 7-10*

To generate constructs that can be used to generate the transgenic mice described herein, the exons containing the promoters for the  $\Delta\text{TRs}$  were cloned. The hinge region:ligand binding domain of GenBank X07751 (nucleotides 360-1357) was used to screen an SV129 lambda fixII genomic library using random primed  $^{32}\text{P}$ -cDNA probes using  
15 methods known in the art (Ausubel et al., eds., 1995, last update April, 2000, Current Protocols in Molecular Biology, John Wiley & Sons, NY). After screening, plaques that hybridized were purified and two clones were selected that contained inserts of 8 kb and 9kb. Restriction mapping revealed that these two clones span exons 6-9 of the thyroid hormone receptor gene and contained intron 7. Similar methods can be used to isolate genomic  
20 sequence encompassing intron 10 from the mouse thyroid hormone receptor gene or from the thyroid hormone receptor genes of other species where cDNA sequences are known.

*Elimination of intron 7 promoters/transcription initiating sequences*

Certain transgenic animals of the invention have the promoters and/or transcription  
25 initiating sequences of intron 7 (in the mouse or the homologous sequences in other animals) altered or eliminated. One method of eliminating the  $\Delta\text{TR}\alpha$  gene products is to remove the promoter region located in intron 7 of the  $\text{TR}\alpha$  gene using homologous recombination. Knock-in of a lox P neo<sup>r</sup> gene selection cassette into intron 7 of the  $\text{TR}\alpha$  gene, is followed by recombinant ES cell selection. The neo<sup>r</sup> selection cassette is then removed thereby restoring  
30 a shortened promoterless intron 7 and the mutant ES cells will be used to generate mice lacking both  $\Delta\text{TR}\alpha\text{s}$  but expressing all full-length  $\text{TR}\alpha$  gene products. Similar methods may be used to alter the intron 7 promoters.

Figure 6 depicts the organization of intron 7 of the mouse  $\text{TR}\alpha$  gene. The AP1, ctf, GR, SP1 and ets-1 sequences of intron 7 have been documented to regulate reporter gene



5 transcription in transient expression assays (Chassande et al., 1997, Mol. Endocrinol. 11: 1278-1290). The isolation of 7-8 kb genomic clones from a mouse 129SV genomic library that span this region is described above.

Because of the lack of unique restriction sites at the 3' end of the intron 7, the 3' thyroid hormone receptor  $\alpha$  gene arm is isolated using PCR-based primers beginning on the 3' end of the SP1 site in intron 7 ( $P_{\text{int7}}[\text{nt}395-414]$  P04-GGAGACTGTGGGGTGTGG; SEQ ID NO: 2). The reverse PCR primer is located in the NEB193 shuttle vector ( $P_{\text{TR}\alpha}$  P04-CCAGTGAATTCGAGCTC-3'; SEQ ID NO: 3) and yields an approximately 3.5 kb fragment. This fragment contains the native intron7/exon 8 splice junction and exon 9 of the thyroid hormone receptor  $\alpha$  gene. Figure 7 illustrates the structure and restriction map of this region as well as the location of the 3' and 5' arms. The 5' arm of the TR $\alpha$  gene is isolated using PstI, yielding a ~4 kb fragment containing the 5' splice junction of intron 7 and the native upstream thyroid hormone receptor  $\alpha$  sequences. The 5' and 3' arms are ligated into the targeting vector Pneotklox (S. Jones, University of Massachusetts Medical Center) to generate a TR $\alpha$  5' arm -loxP neo<sup>r</sup> loxP-TR $\alpha$  3' arm; the negative selection marker TK is appended to the MCS located at the 3' end of the 3' arm of the TR $\alpha$  gene. Restriction mapping and cycle sequencing is used to confirm construct integrity. LoxP sites bracket the G418 resistance gene allowing elimination of this selection marker from ES cells after homologous recombination by transient expression of Cre-expressing plasmids in recombinant ES cells. Regeneration of a shortened intron 7 lacking any coding sequences will eliminate troublesome effects of intron-based coding sequences on expression of the recombinant TR $\alpha$  gene. Other suitable vectors may be used.

#### *Deletion of TR $\alpha$ exon 10*

A second knockout construct targets exon 10 of the TR $\alpha$  gene. The rationale for this knockout is based on the finding that the TRs differ only in the C-terminus (encoded in the mouse in exon 10) and TR $\alpha$ 1 is not found in myosin V-enriched synaptic vesicles. Thus,

TR $\alpha$ 2 is the prime candidate as TH-dependent link between the synaptic vesicles to myosin V. Deletion of exon 10 eliminates the ability to produce both full-length, non-T3 binding TR $\alpha$ 2 and TR $\alpha$ 2. These mice retain expression of the T3-binding thyroid hormone

5 receptors that regulate gene expression and the  $\Delta TR\alpha 1$ , and thus allow examination of the  
role of  $\Delta TR\alpha 2$  and full-length  $TR\alpha 2$ . The  $TR\alpha$  clone described above spans this region and  
the entire sequence of exon 10 is replaced with the  $neo^r$  selection gene using standard  
approaches and appropriate restriction sites. Figure 8 illustrates the structure and restriction  
map of this region. It is not necessary in the case of exon 10 deletions to remove the  $Neo^r$   
10 selection gene prior to homologous recombination in ES cells.

ES cells are electroporated with *PacI* linearized *int7* or *exon10* targeting constructs,  
grown, and recombinant ES cells are isolated using both positive ( $neo^r$ ) and negative (TK)  
selection according to established protocols (e.g., Ausubel et al., *supra*). Depending on the  
targeting construct, this results in the selection of  $ES^{int7+/-}$  and the  $ES^{exon10+/-}$  cells. Restriction  
15 fragment polymorphism (RFP) analysis and standard Northern analysis of the candidate  
recombinant ES cells is used to confirm the presence of the appropriate  $TR\alpha$  gene mutations.

#### *Production and mouse husbandry*

Injection of targeted ES clones ( $ES^{int7+/-}$  and the  $ES^{exon10+/-}$ ) into mouse blastocytes is  
20 performed for each mutation in duplicate to insure success in the event that any individual ES  
clone does not contribute to the germline. Identification of founder mice is performed using  
standard RFP analysis of tail DNA. As is the case with *dilute-lethal* heterozygotes, for both  
the  $\Delta intron7+/-$  and the  $\Delta exon10+/-$  founders, one normal allele is expected to provide a  
normal phenotype and the progeny of heterozygote breeding should yield typical Mendelian  
25 patterns. As seen in the *dilute-lethal* homozygotes, failure to gain weight, disturbances in  
motor and sensory function, and most likely death will occur prior to maturation at 21 to 28  
days. Expression of the  $\Delta TRs$  is monitored by Northern blot, and immunoblot analysis of  
whole brain and myosin V-enriched synaptic vesicles.

Functional analysis of the effect of losing the  $\Delta TRs$  on actin-based endocytosis is  
30 performed using cultured astrocytes prepared from affected and normal progeny. As with the  
*dilute-lethal* phenotype, disturbed motor function is a potential indicator of the homozygous  
phenotype. If such a physical sign is lacking, or as an additional test, astrocytes are prepared  
from individual progeny and the TH-dependent endocytosis of D2 monitored according to  
established procedures and procedures described herein.

5 One use of the transgenic animals described above is that they can be used to evaluate the role of TR $\alpha$ s in whole animal viability and in actin-based endocytosis. Furthermore, such animals can be used to evaluate the role of this non-genomic thyroid hormone effector molecule in synapse function. Such animals can also be used to test the effects of candidate compounds in the absence of a receptor, e.g., on non-thyroid hormone receptor associated effects.

Both  $\Delta$ TR $\alpha$ 1 and  $\Delta$ TR $\alpha$ 2 are expressed in the developing and adult brain. The loss of activity of either or both of these gene products will lead to unproductive or impaired synaptic vesicle recycling and reduced synaptic function.

#### 15 Example 6: Screening Assays for TR $\alpha$ Ligands that Modulate Actin-Based Endocytosis

A number of different assays can be used to identify the candidate compounds of the invention. Examples of such assays are below.

##### *Identification of $\Delta$ TR $\alpha$ ligands using direct ligand binding assays*

20 One assay principle is based on the ligand-dependent association of vesicles to microfilament bound myosin V. As discussed above, immunoreactive  $\Delta$ TR $\alpha$ 2 appears to be constitutively bound to the extreme C-terminus of myosin V and the addition of specific thyroid hormones (T4 and rT3 but not T3) cause membrane vesicles to bind to the microfilaments. Thus, ligand binding serves to tether a vesicle to the actin-bound myosin V.

25 Deletion studies (*supra*) have identified the last 22 amino acids of myosin V as the region responsible for tethering the vesicle to myosin V. The use of actin and myosin complexes can be eliminated by directly fixing a  $\Delta$ TR $\alpha$  to a solid phase or by fixing a peptide derived from the C-terminus of myosin V to a solid phase and allowing the  $\Delta$ TR $\alpha$  to bind to this specific peptide. Ligand-dependent binding of labeled (e.g., with a fluorescent molecule)

30 vesicles to the immobilized  $\Delta$ TR $\alpha$  can be monitored directly by high throughput readers that detect the label.

For example, N-tagged (6-his or HA)  $\Delta$ TR $\alpha$  are fixed to a solid matrix such as a microtiter dish or nitrocellulose membrane by Ni chelation. Alternatively, antisera raised against the epitope tag (6-His, HA or c-myc) can be fixed to the surface using standard

5 procedures and the eiptope tagged  $\Delta\text{TR}\alpha$  adsorbed to the immobilized antibody. The  
resultant immobilized  $\Delta\text{TR}\alpha$  can be incubated with ligand, flavonoids, arones, thyroid  
hormone analogs, or other test or candidate compounds in the presence of membrane vesicles  
containing a covalently integrated fluorescent tag. After washing, the fluorescence intensity  
10 remaining on the solid surface is determined. Avidity, potency and competition with thyroid  
hormones can be tested using this approach.

#### *Vesicle motion studies with T4 and rT3*

Another assay that can be used in the invention is based on the observation that  
specific, thyroid hormone-dependent actin-based endocytosis can be visualized in real time in  
15 living astrocytes. Analysis of the hormone-dependent internalization of vesicles can be used  
to evaluate the potency and specificity of specific  $\Delta\text{TR}\alpha$  ligands on actin-based endocytosis  
in living cells. To perform such assays, for example, astrocytes constitutively expressing a  
green-fluorescent protein (GFP)-D2 fusion protein are grown in hormone-free medium on  
cover slips and treated as described in Leonard, 1988, Biochem. Biophys. Res. Comm.  
20 151:1164-1172 and Leonard et al., 1990, J. Biol. Chem. 265: 940-946. Baseline vesicle  
movement during a 10 minute initial period will be collected using digital imaging  
microscopy. Next, a candidate compound identified in the ligand binding screening phase is  
added to the cells. Internalization of fluorescent D2 over a 10 minute period is monitored,  
and the data collection and three dimensional reconstruction is completed. This assay can  
25 provide an analysis of the potency of a candidate compound in initiating actin-based  
endocytosis. As with the other assays, this assay may also be used to initially identify  
candidate compounds.

Alternatively, end point assays can be done using the initial and final distribution of  
fluorescent D2. Instead of D2, other vesicle proteins such as synaptotagmin, synaptophysin  
30 can serve as the analyte.

#### *D2 measurement as a marker in vivo and in culture*

There is rapid hormone-dependent regulation of the biological half-life of D2 in  
cAMP-stimulated astrocytes. This actin-based endocytotic event occurs in the brain *in vivo*

5 and in cAMP-stimulated astrocytes in culture. Direct analysis of the D2 levels in the brain and/or in cultured astrocytes is another biological end-point for evaluating the selectivity, avidity, and potency of candidate compounds (e.g.,  $\Delta\text{TR}\alpha$  ligands). This assay is based on the rapid down-regulation of D2 activity after acute hormone treatment of thyroid hormone-deficient rats (Silva and Leonard, 1985, *Endocrinol.* 116:1627-1635). For example, to  
10 perform such an assay in rodents, animals are rendered thyroid hormone deficient by administration of anti-thyroid drugs such as propylthiouracil (PTU) or methylmercaptoimidazole (MMI) for 2 weeks prior to study (Farwell and Dubord-Tomasetti, 1999, *Endocrinol.* 140:4221-4227). Test  $\Delta\text{TR}\alpha$  ligands are administered either by ip or iv routes and the levels of D2 activity in brain homogenates determined using assays known in  
15 the art (Leonard and Rosenberg, 1980, *Endocrinol.* 107:1376-83; Visser et al., 1982, *Proc. Nat. Acad. Sci. USA* 79:5080-5084).

D2 activity-based assays can also be performed in cultured cells. For example, in cAMP-stimulated astrocytes grown in serum-free medium, acute thyroid hormone replacement leads to the rapid (within 10-20 minutes) fall in D2 levels (Leonard, 1988,  
20 *supra*; Leonard et al., 1990, *supra*; Safran et al., 1993, *supra*). To perform the assay, confluent monolayers of cultured astrocytes are grown in serum-free medium for 24 hours and D2 activity is induced with 1 mM  $\text{bt}_2\text{cAMP}$  and 100 nM hydrocortisone for 16 hours. Candidate ligands are added in increasing concentrations, the cells harvested after 20 minutes, and D2 activity determined.

25 D2 activity can be determined using known methods. For example, D2 activity is determined by measuring the release of radioiodide from 2 nM [ $^{125}\text{I}$ ] 3' or 5'-rT3 in the presence of 20 mM dithiothreitol and 1 mM 6-n-propylthiouracil (PTU) (Leonard, 1988, *supra*; Leonard et al., 1981, *Science* 214:571-573; Leonard and Rosenberg, 1978, *Endocrinol.* 103:2137-2144; Visser et al., 1981, *Biochem. Biophys. Res. Comm.* 101:1297-  
30 304; Visser et al., 1982, *supra*). Assays are done in a total volume of 100  $\mu\text{l}$ . 20–100  $\mu\text{g}$  of cell or tissue homogenate protein is added to an assay mixture composed of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 2 nM [ $^{125}\text{I}$ ] 3' or 5'-rT3 (500 cpm/fmol), 20 mM dithiothreitol and 1 mM PTU. After incubation at 37 °C for 20-60 minutes, 50  $\mu\text{l}$  of a stop solution (4 mg/ml BSA and 5 mM PTU) that binds rT3 and prevents re-organization of

5 the released radioiodide is added and the unmetabolized substrate is precipitated with 350  $\mu$ l of 10% trichloroacetic acid (TCA; w/v). After clarification by centrifugation, the acid soluble extract containing the released radioiodide is passed over a 2 ml bed of cation exchange resin (DOWEX 50W) to remove any unprecipitated rT3 and the flow through fraction is counted in a well-type  $\gamma$  counter.

10

*In vitro actin binding assay: thyroid hormone-dependent binding of p29 vesicles to the F-actin cytoskeleton*

An *in vitro* binding assay was developed whose principle is based on the hormone dependent redistribution of affinity labeled p29 between the Triton-soluble (vesicle pool) and  
15 Triton-insoluble (F-actin cytoskeleton) fractions. The assay was used to examine the hormone dependent relationship between the p29-containing endosome and the F-actin cytoskeleton. Two different pools of astrocytes were prepared. *F-lysate* provided a fully polymerized F-actin cytoskeleton with its associated myosin V and was prepared by treating thyroid hormone-deficient astrocytes in serum-free medium for 24 hours with 10  $\mu$ M retinoic  
20 acid (Farwell and Leonard, 1992, Endocrinol. 131: 721-728). The other astrocyte pool was used to prepare affinity BrAc [ $^{125}$  I]T4-labeled p29 vesicles from thyroid-deficient cells (*V-lysate*; Farwell et al. 1990, J. Biol. Chem. 265:18546-18553). *V-lysates* were prepared from cAMP-stimulated astrocytes grown in serum-free media that were labeled with 2 nM BrAc[ $^{125}$  I]T4 as described in Farwell et al. (1990, J. Biol. Chem. 265:18546-18553).  
25 Microtubules were eliminated in all cells by treatment with 10  $\mu$ M colchicine for 30 minutes prior to cell isolation. After pretreatment, cells were scraped from the flask, collected by centrifugation (500 x g for 5 minutes), washed with phosphate buffered saline (pH 7.4), and the cell pellets were lysed by two freeze-thaw cycles (Farwell et al., 1990, *supra*; Farwell et al., 1993, J. Biol. Chem. 268:5055-5062). Lysates could be stored at -70°C for up to 4 weeks  
30 without loss of biological activity.

To perform actin attachment assays, *F-* and *V-lysates* (100  $\mu$ g cell protein each) were combined on ice, then 10 nM T4, 10 nM rT3, or 10 nM T3 were added, and the mixtures were incubated for 20 minutes at 37 ° C. After the incubation, the mixtures were chilled on ice for 2 minutes, then Triton X-100® (0.5% v/v, final) was added and the soluble (Triton

5 supernatant, vesicles) and insoluble (Triton pellet, F-actin) fractions were separated by microfuge centrifugation at 4°C for 5 minutes. The distribution of [<sup>125</sup>I]-labeled p29 was then determined by SDS PAGE analysis.

This assay permits analysis of the effects of thyroid hormone on the distribution of [<sup>125</sup>I]-labeled p29 vesicles. In these experiments, comparable amounts of radiolabeled p29  
10 were present in all treatment groups, as judged by the intensity of the lower band of the doublet of radiolabeled proteins at about 30 kDa (Farwell and Leonard, 1989, J. Biol. Chem. 264:20561-20567). Addition of 10 nM T3 to the mixed cell lysates did not lead to the binding of [<sup>125</sup>I]-labeled p29 to F-actin and more than 90% of the p29 remained in the non-actin bound, Triton-soluble fraction. Addition of 10 nM T4 to the broken cell preparation  
15 resulted in the binding of more than 70% of the radiolabeled p29 to the Triton insoluble, F-actin fraction. These data indicate that the T4-dependent binding of the p29 subunit of D2 to the actin cytoskeleton observed in living astrocytes (Farwell et al. 1990, J. Biol. Chem. 265: 18546-18553) is mimicked in broken cell preparations.

These data demonstrate that this *in vitro* actin binding assay can be used to assay test  
20 compounds and candidate compounds and is useful for the present invention.

*Binding of p29 to the actin cytoskeleton is calcium, magnesium, and ATP dependent*  
The *in vitro* actin binding assay demonstrated that T4 specifically induced the binding of p29  
endosomes to F-actin. To determine whether this is a direct interaction between the vesicle  
and F-actin or is mediated by other actin-bound proteins, e.g., myosin V, the release of p29  
25 vesicle bound to F-actin by activating a Ca-dependent Mg-ATPase was investigated. Synaptic vesicle-bound myosin V is released from F-actin by activating the Ca-dependent MgATPase found in the actin binding head of myosin V (Prekeris et al, 1997, J. Cell Biol. 137: 1589-1601; Nascimento et al., 1996, J. Biol. Chem. 271:17561-17569). In this experiment, confluent monolayers of astrocytes were grown in serum-free media for 16 hours, then D2  
30 activity was induced with bt<sub>2</sub>cAMP and hydrocortisone, and the p29 vesicle was affinity radiolabeled with BrAc[<sup>125</sup>I]T4. Cell lysates containing approximately 50,000 cpm of BrAc[<sup>125</sup>I]T4-labeled p29 were pretreated with 10nM for 20 minutes at 37°C, followed by an additional 20 minute incubation with 0.1 mM Ca<sup>2+</sup>, 1mM Mg<sup>2+</sup>, 0.1 mM ATP, or 5 mM EGTA. Triton-insoluble (F-actin bound) pellets were prepared as described herein.

5 Equal volumes of *V-lysate* containing BrAc[<sup>125</sup>I]T4-labeled p29 vesicles and *F-lysate* were mixed and maximal p29 vesicle:F-actin binding was initiated by adding 10 nM T4. Following pre-incubation with T4, the reconstituted lysates were then supplemented with 0.1 mM Ca<sup>2+</sup>, 1 mM Mg<sup>2+</sup>, 0.1mM ATP, and/or 5 mM EGTA, and incubated for 30 minutes at 37°C. Triton-insoluble (F-actin-bound) pellets were separated from the Triton-soluble (vesicle) fraction by microfuge centrifugation (14,000 x g for five minutes) and the distribution of p29 was determined by  $\gamma$ -counting, and by SDS-PAGE analysis.

Approximately 80% of the total p29 vesicles added were bound to F-actin at the start of the experiment. Addition of divalent ions (Ca<sup>2+</sup> and Mg<sup>2+</sup>) and ATP resulted in the release of about 70 % of the p29 vesicles from F-actin without altering the F-actin content in the Triton pellet. The calcium chelator, EGTA, blocked more than 50% of the Ca<sup>2+</sup>-stimulated, Mg<sub>2</sub>ATPase-dependent release of p29 from F-actin. Similarly, removing the substrate, ATP, either divalent ion (Mg<sup>2+</sup>, or Ca<sup>2+</sup>) completely blocked the release of p29 vesicles from the actin cytoskeleton. These data show that F-actin-bound, myosin motor protein(s), presumably myosin V, participate in the binding of the p29 vesicle to F-actin.

#### Example 7: Determination of Dose-Response Relationships of $\Delta$ TR $\alpha$ ligands

##### *Large scale screening*

Both dose-response relationships and competition assays are determined for likely  $\Delta$ TR $\alpha$  ligands (candidate compounds) identified, e.g., by initial screens. Increasing concentrations of a candidate compound are added to a fixed concentration of an immobilized  $\Delta$ TR $\alpha$  and the fluorescent vesicle and the ligand-dependent binding of the analyte vesicles systematically determined. From these data K<sub>a</sub> (EC50's) can be determined.

Competition assays for the immobilized  $\Delta$ TR $\alpha$  with T4, the presumed physiological ligand, are performed by simple radiometric assay. Immobilized  $\Delta$ TR $\alpha$  is incubated with 1 nM [<sup>125</sup>I]-T4 and increasing concentrations of candidate  $\Delta$ TR $\alpha$  ligands for 30 to 120 minutes at 4°C. Unbound [<sup>125</sup>I]-T4 is removed by repeated washing and the quantity of [<sup>125</sup>I]-T4 bound to the immobilized  $\Delta$ TR $\alpha$  determined. Relative potency for the binding site, and specificity of binding can be characterized using these approaches.



Based on the competitive binding curves and dose-response relationships determined using the simple binding assays described herein, the ability of the candidate compounds, e.g.,  $\Delta\text{TR}\alpha$  ligand(s) to initiate actin-based endocytosis, and D2 inactivation is determined using methods known in the art (Leonard et al., 1990, J. Biol. Chem. 265:940-946; Safran et al., 1993, J. Biol. Chem. 268:14224-14229) and described herein.

Example 8: Hormone-induced Synaptic Vesicle (SV) Trafficking in Cerebellar Granule Neurons

All of the major components of hormone-induced vesicle trafficking in astrocytes (actin fibers, myosin V (also referred to as myosin 5a and myo 5a), and recycling vesicles) are also present in nerve terminals. Therefore, the effects of thyroid hormone on synaptic vesicle recycling in the presynaptic nerve terminal were examined using myo5a-dependent synaptic vesicle (SV) recycling in cultured cerebellar granule neurons. In these experiments, rat granule neurons were grown on laminin-coated coverslips in the absence or presence of hormone and synaptic vesicle recycling was examined using FM1-43 (a styryl dye used in the art for studying endocytosis and exocytosis, e.g., in synaptic vesicle uptake and release assays; Molecular Probes, Inc. Eugene, OR). These experiments demonstrated that K<sup>+</sup>-induced synaptic vesicle loading was under hormonal control. Analysis of the quantity of FM1-43 that was in the reserve pool, i.e., retained by individual nerve terminals after a three minute period of autonomous activity, showed that >80% of the dye was released from granule neurons grown in hormone-free medium. In contrast, granule neurons grown in thyroid hormone supplemented medium released only 15-20% of the FM1-43 after six minutes of incubation and the hormone specificity of retention of FM1-43 in the "reserve pool" was identical to that determined for myo5a-dependent, endocytosis of p29 vesicles (D2p29 vesicles) in astrocytes.

To perform these experiments, cultured cerebellar granule neurons were used to determine if recycling synaptic vesicles showed hormone-induced myo5a-dependent trafficking. Cerebellar granule neurons were examined because thyroid hormone has a profound influence on granule neuron viability during cerebellar maturation (Xiao and Nikodem, 1998, Front. Biosci. 3: A52-57; Rabie et al., 1980, Brain Res. 190:409-414), and

- 5 because of the effects of the loss of myo5a on synaptic vesicle homeostasis (Bridgman, 1999, J. Cell Biol. 146:1045-1060). Cerebellar granule neurons were isolated from 4 day old rats, seeded at  $\sim 100,000$  cells/cm<sup>2</sup> on p-lysine/laminin-coated coverslips and grown for 7-10 days in BME medium supplemented with 10% (v/v) fetal calf serum. Astrocyte proliferation was suppressed by treating the cultures on day three and day six with the anti-mitotic, Ara-C.
- 10 The cultures contained multi-processed, neurofilament positive neurons and a few polygonal astrocytes.

Experiments were conducted to determine whether synaptic vesicle endocytosis could be altered by the hormone status of the neuron. Recycling synaptic vesicles were labeled with FM1-43. Synaptic vesicle recycling was initiated by depolarization with 50 mM KCl

15 for 60 seconds. Cells were then rapidly washed free of KCl and the endocytic synaptic vesicles were then loaded by a 60 second incubation with 15 mM FM1-43 in the absence of KCl. The dye was then removed and the number, intensity and retention of the FM1-43 in random nerve terminals immediately after washing and after six minutes of autonomous

20 activity were analyzed in paraformaldehyde fixed cells (Ryan, 1999, J. Neurosci.: 19:1317-1323). Fluorescence in single nerve terminals (ROI=7 x 7 pixels) and in an adjacent segment of the neuronal fiber were measured for 40-45 individual nerve terminals. Images were captured using Nikon Eclipse E600 microscope, fitted with a 40X/0.75 Plan Fluor objective and a SPOT RT Color digital camera. Fluorescence intensity was determined using NIH

25 Image software. The total FM1-43 fluorescence in a nerve terminal represents the sum of the dye-loaded synaptic vesicles in the readily releasable and the reserve pools.

Under the labeling conditions described above, recycling vesicles in both neurons and astrocytes took up the dye; however, the neurons showed the punctate pattern of staining characteristic of functional synapses, i.e., bright spots distributed along long fibers and on the surface of the nerve cell body. By allowing recurrent activity, the FM1-43 filled synaptic

30 vesicles present in the readily releasable pool will be progressively depleted. Thus, the residual FM1-43 found in nerve terminals after six minutes of autonomous activity should represent the recycled synaptic vesicles that were shuttled to the reserve pool. By measuring the total FM1-43 content in random nerve terminals immediately after loading and at 30 second intervals for six minutes of spontaneous activity, the influence of hormones on the

5 distribution of recycling synaptic vesicles between the readily releasable and reserve pools was estimated.

The data in Figure 10 show the effects of T3 and T4 on the FM1-43 content in nerve terminals of cultured cerebellar neurons immediately after loading and after three minutes of spontaneous activity. No differences in the uptake of FM1-43 were observed in the three  
10 experimental conditions tested indicating that hormones did not affect the initial recovery of synaptic vesicles from the plasma membrane. After three minutes of spontaneous activity, >80% of the endocytosed FM1-43 was released in both the no hormone and the T3-treated neurons. This suggests that most of the FM1-43 filled synaptic vesicles had remained in the readily releasable pool. In contrast, granule neurons acutely treated with T4 retained >90%  
15 of the endocytosed FM1-43 after three minutes of spontaneous stimulation. These data suggest that T4, but not T3, promoted the centripetal redistribution of the newly recaptured synaptic vesicles from the readily releasable pool to the reserve pool, just as was found for endocytosis of recycling vesicles in astrocytes.

#### 20 Example 9: Dynamic Studies of Synaptic Vesicle Recycling

To examine the dynamics of synaptic vesicle recycling and the role of thyroid hormone in the process, neurons expressing selected dominant negative myo5a constructs were used.

Granule neurons were grown for 7 days and rendered hormone free by growth in  
25 serum-free medium as detailed above. Coverslips were then placed in a perfusion chamber, mounted on the stage of a laser scanning confocal microscope and perfused at 1 ml/min at 25°C with perfusion buffer (119 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 25 mM HEPES (pH 7.4), 30 mM glucose, and 50 mM AP-5). The effects of different hormones on synaptic vesicle trafficking were then determined. In brief, neurons were  
30 perfused with labeling buffer consisting of perfusion buffer containing BSA buffered 10 nM T4, 10 nM T3, or 10 nM rT3; 15 mM FM1-43; and 50 mM KCl for one minute. Depolarizing KCl was then removed from the perfusion buffer and the cells were exposed to FM1-43 for an additional 30 seconds to insure complete loading of recycling synaptic vesicles. Loaded cells were then washed free of dye and the cells were perfused for  
35 ten minutes at rest.

5 Images of a single field of neuronal fibers were then taken immediately before adding  
a second depolarizing solution (perfusion buffer containing 50 mM KCl) and at one and two  
minutes after beginning the continuous stimulation by 50 mM KCl. Thirty to forty-five  
individual FM1-43 labeled nerve terminals were identified in the baseline images (taken prior  
to depolarization) and the change in fluorescence (DF) for each nerve terminal determined at  
10 the one and two minute time points. Figures 11A and 11B show the results of analysis of the  
hormone specificity of synaptic vesicle trafficking (Fig. 11A) and the influence of individual  
myo5a mutants (Fig. 11B) on synaptic vesicle transport in granule neurons.

As observed in the synaptic vesicle labeling study of astrocytes, hormones that  
initiate actin-based endocytosis of recycling vesicles in astrocytes (i.e., T4 and rT3) also  
15 promote the retention of FM1-43 labeled synaptic vesicles even during a maximal  
depolarizing stimulus. Assuming first order kinetics for the release of endocytosed FM1-43  
during continuous stimulation, both T4 and rT3 decreased the rate of FM1-43 release by 75%  
( $t_{1/2}$ = 182 seconds vs. 44 seconds in no hormone controls). This is in contrast to T3 which  
had no effect on the retention of recycling synaptic vesicles ( $t_{1/2}$ = 44 seconds). These results  
20 indicate that the delivery of recycling synaptic vesicles to the nonreleasable or reserve pool in  
the presynaptic nerve terminals of cerebellar granule cells shows the same hormone  
specificity as the recycling vesicles in rat astrocytes.

These data show that candidate compounds of the invention that are, e.g., discovered  
using astrocyte assays, can also be effective in neuronal cells.

25 In a second study, the dynamics of synaptic vesicle recycling in individual nerve  
terminals using the loading-rest-stimulation paradigm of Ryan (Ryan et al, 1996, J. Cell Biol.  
134:1219-1227; Ryan, 1999, J. Neurosci. 19:1317-1323; Ryan et al, 1993, Neuron 11: 713-  
724; Sankaranarayanan and Ryan, 2000, Nat. Cell Biol. 2:197-204) were studied.

To evaluate the role of myo5a in synaptic vesicle transport, three different myo5a  
30 constructs were introduced into the neurons by replication-deficient adenoviral vectors  
( $\Delta$ myo5a<sup>tail</sup> (MyoV<sup>tail</sup>),  $\Delta$ myo5a<sup>C-term</sup> (MyoV<sup>1830</sup>) and  $\Delta$ Myo5a<sup>CC</sup> (MyoV<sup>CC</sup>) see Figure 5) as  
described above. The granule neurons were readily infected with Ad5 constructs as was  
observed for cultured rat astrocytes. Neurons were infected with purified Ad5-constructs  
(MOI=10) 48 hours prior to the synaptic vesicle trafficking study, and allowed to recover in  
35 hormone (T4) supplemented medium. As detailed in Figure 11B, over-expression of the

5 ~88kDa myo5a tail (myo5a<sup>tail</sup>) completely blocked the hormone-dependent retention of FM1-43 loaded synaptic vesicles, while expression of the coiled-coil region ( $\Delta$ myo5a<sup>CC</sup>) had little, if any, effect on the retention of recycled synaptic vesicles in nerve terminals exposed to continuous depolarization. Expression of the vesicle-binding domain (VBD,  $\Delta$ myo5a<sup>C-term</sup>) had a modest but statistically significant effect and increased the  $t_{1/2}$  of FM1-43 retention by 10 50%. This modest influence of the VBD mutant on synaptic vesicle retention is likely to be due to the loss of the kinesin-binding domain that limits its delivery to nerve terminals. .

These results confirm the specific hormone-dependent delivery of FM1-43-loaded synaptic vesicles to a "reserve pool" and show that over-expression of the dominant negative myo5a<sup>tail</sup> specifically blocks FM1-43 retention by these neurons. Thus, it appears that myo5a 15 delivers the recycled synaptic vesicles to a reserve pool and that these synaptic vesicles can be mobilized under prolonged stimulation. These data point directly to myo5a as the motor responsible for synaptic vesicle recycling in cerebellar granule neurons and indicate that this process is similar to the myo5a-dependent vesicle trafficking in astrocytes. These results suggest that the endocytosed synaptic vesicle in the presynaptic nerve terminal binds to the 20 vesicle binding domain of the myo5a tail and is then delivered to the reserve pool, where synapsin-dependent crosslinks immobilize the synaptic vesicle and stabilize the reserve pool. Without the myo5a-dependent vectorial delivery of the synaptic vesicles to the reserve pool, a key communication pathway is lost and this interrupted reciprocity between the two pools of synaptic vesicles leads to synaptic fatigue.

25 These data show that myo5a is a target for identifying compounds that modulate synaptic vesicle trafficking. The data also suggest that transport of recycling synaptic vesicles to the reserve pool in the nerve terminal can be modulated by hormones, therefore, analogs and antagonists of such hormones can be useful for modulating synaptic vesicle transport, particularly synaptic vesicle trafficking between the active and the reserve zones of 30 synaptic vesicle pools. Such hormones, analogs, and antagonists are useful for treating disorders or conditions of the nervous system that can be treated by altering synaptic vesicle trafficking, thus affecting the synthesis, storage, release, or degradation of a neurotransmitter.

**OTHER EMBODIMENTS**

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

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